Exhibit 1

GNE.3230R1C39

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Eaton, et al.
Appl. No.	:	10/063,557
Filed	:	May 2, 2002
For	:	SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING THE SAME
Examiner	:	David J. Blanchard
Group Art Unit	:	1642

DECLARATION OF J. CHRISTOPHER GRIMALDI, UNDER 37 CFR §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, J. Christopher Grimaldi, declare and state as follows:

1. I am a Senior Research Associate in the Molecular Biology Department of Genentech, Inc., South San Francisco, CA 94080.

2. My scientific Curriculum Vitae, including my list of publications, is attached to and forms part of this Declaration (Exhibit A).

3. I joined Genentech in January of 1999. From 1999 to 2003, I directed the Cloning Laboratory in the Molecular Biology Department. During this time I directed or performed numerous molecular biology techniques including semi-quantitative Polymerase Chain Reaction (PCR) analyses. I am currently involved, among other projects, in the isolation of genes coding for membrane associated proteins which can be used as targets for antibody therapeutics against cancer. In connection with the above-identified patent application, I personally performed or directed the semi-quantitative PCR gene expression analyses in the assay entitled "Tumor Versus Normal Differential Tissue Expression Distribution," which is described in EXAMPLE 18 in the specification. These studies were used to identify differences in gene expression between tumor tissue and their normal counterparts.

4. EXAMPLE 18 reports the results of the PCR analyses conducted as part of the investigating of several newly discovered DNA sequences. This process included developing

-1-

primers and analyzing expression of the DNA sequences of interest in normal and tumor tissues. The analyses were designed to determine whether a difference exists between gene expression in normal tissues as compared to tumor in the same tissue type.

5.   The DNA libraries used in the gene expression studies were made from pooled samples of normal and of tumor tissues. Data from pooled samples is more likely to be accurate than data obtained from a sample from a single individual. That is, the detection of variations in gene expression is likely to represent a more generally relevant condition when pooled samples from normal tissues are compared with pooled samples from tumors in the same tissue type.

6.   In differential gene expression studies, one looks for genes whose expression levels differ significantly under different conditions, for example, in normal versus diseased tissue. Thus, I conducted a semi-quantitative analysis of the expression of the DNA sequences of interest in normal versus tumor tissues. Expression levels were graded according to a scale of +, - , and +/- to indicate the amount of the specific signal detected. Using the widely accepted technique of PCR, it was determined whether the polynucleotides tested were more highly expressed, less expressed, or whether expression remained the same in tumor tissue as compared to its normal counterpart. Because this technique relies on the visual detection of ethidium bromide staining of PCR products on agarose gels, it is reasonable to assume that any detectable differences seen between two samples will represent at least a two fold difference in cDNA.

·7.   The results of the gene expression studies indicate that the genes of interest can be used to differentiate tumor from normal. The precise levels of gene expression are irrelevant; what matters is that there is a relative difference in expression between normal tissue and tumor tissue. The precise type of tumor is also irrelevant; again, the assay was designed to indicate whether a difference exists between normal tissue and tumor tissue of the same type. If a difference is detected, this indicates that the gene and its corresponding polypeptide and antibodies against the polypeptide are useful for diagnostic purposes, to screen samples to differentiate between normal and tumor. Additional studies can then be conducted if further information is desired.

8.   I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

By: _____   Date:___ 8/10/2004 ___
        J Christopher Grimaldi

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J. Christopher Grimaldi

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San Francisco, CA 94122
(415) 681-1639 (Home)

EDUCATION

University of California, Berkeley
Bachelor of Arts in Molecular Biology, 1984

EMPLOYMENT EXPERIENCE

SRA Genentech Inc., South San Francisco; 1/99 to present

Previously, was responsible to direct and manage the Cloning Lab. Currently focused on isolating cancer specific genes for the Tumor Antigen (TAP), and Secreted Tumor Protein (STOP) projects for the Oncology Department as well as Immunologically relevant genes for the Immunology Department. Directed a lab of 6 scientists focused on a company-wide team effort to identify and isolate secreted proteins for potential therapeutic use (SPDI). For the SPDI project my duties were, among other things, the critically important coordination of the cloning of thousands of putative genes, by developing a smooth process of communication between the Bioinformatics, Cloning, Sequencing, and Legal teams. Collaborated with several groups to discover novel genes through the Curagen project, a unique differential display methodology. Interacted extensively with the Legal team providing essential data needed for filing patents on novel genes discovered through the SPDI, TAP and Curagen projects. My group has developed, implemented and patented high throughput cloning methodologies that have proven to be essential for the isolation of hundreds of novel genes for the SPDI, TAP and Curagen projects as well as dozens of other smaller projects.

Scientist DNAX Research Institute, Palo Alto; 9/91 to 1/99

Involved in multiple projects aimed at understanding novel genes discovered through bioinformatics studies and functional assays. Developed and patented a method for the specific depletion of eosinophils in vivo using monoclonal antibodies. Developed and implemented essential technical methodologies and provided strategic direction in the areas of expression, cloning, protein purification, general molecular biology, and monoclonal antibody production. Trained and supervised numerous technical staff.

Facilities Manager Corixa, Redwood City; 5/89 - 7/91.

Directed plant-related activities, which included expansion planning, maintenance, safety, purchasing, inventory control, shipping and receiving, and laboratory management. Designed and implemented the safety program. Also served as liaison to regulatory agencies at the local, state and federal level. Was in charge of property leases, leasehold improvements, etc. Negotiated vendor contracts and directed the purchasing department. Trained and supervised personnel to carry out the above-mentioned duties.

SRA   University of California, San Francisco
                  Cancer Research Institute; 2/87-4/89.

Was responsible for numerous cloning projects including: studies of somatic hypermutation, studies of AIDS-associated lymphomas, and cloning of t(5;14), t(11;14), and t(8;14) translocations. Focused on the activation of hemopoietic growth factors involved in the t(5;14) translocation in leukemia patients..

Research
Technician   Berlex Biosciences, South San Francisco; 7/85-2/87.

Worked on a subunit porcine vaccine directed against Mycoplasma hyopneumoniae. Was responsible for generating genomic libraries, screening with degenerate oligonucleotides, and characterizing and expressing clones in E. coli. Also constructed a general purpose expression vector for use by other scientific teams.

PUBLICATIONS

1. Hilary F. Clark, et al. "The Secreted Protein Discovery Initiative (SPDI), a Large-scale Effort to Identify Novel Human Secreted and Transmembrane Proteins: a bioinformatics assessment." Genome Res. Vol 13(10), 2265-2270, 2003

2. Sean H. Adams, Clarissa Chui Sarah L. Schilbach, Xing Xian Yu, Audrey D. Goddard, J. Christopher Grimaldi, James Lee, Patrick Dowd, David A. Lewin, & Steven Colman BFIT, a Unique Acyl-CoA Thioesterase Induced in Thermogenic Brown Adipose Tissue: Cloning, organization of the humanb gene and assessment of a potential link to obesity" Biochemical Journal, Vol 360, 135-142, 2001

3. Szeto W, Jiang W, Tice DA, Rubinfeld B, Hollingshead PG, Fong SE, Dugger DL, Pham T, Yansura D, Wong TA, Grimaldi JC, Corpuz RT, Singh JS, Frantz GD, , Devaux B, Crowley CW, Schwall RH, Eberhard DA, Rastelli L, Polakis P; and Pennica D. "Overexpression of the Retenoic Acid-Responsive Gene Stra6 in Human Cancers and its Synergistic Activation by Wnt-1 and Retinoic Acid." Cancer Research Vol. 61(10), 4197-4205, 2001

4. Jeanne Kahn, Fuad Mehraban, Gladdys Ingle, Xiaohua Xin, Juliet E. Bryant, Gordon Vehar, Jill Schoenfeld, J. Christopher Grimaldi (incorrectly named as "Grimaldi, CJ"), Franklin Peale, Aparna Draksharapu, David A. Lewin, and Mary E. Gerritsen. "Gene Expression Profiling in an in Vitro Model of Angiogenesis." American Journal of Pathology Vol 156(6), 1887-1900, 2000.

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9. M. J. Guimaraes, J. F. Bazan, A. Zolotnik, M. V. Wiles, J. C. Grimaldi, F. Lee, T. McClanahan. "A new approach to the study of haematopoietic development in the yolk sac and embryoid body." Development, Vol. 121(10), 3335-3346, 1995

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11. Leopoldo Santos-Argumedo, Frances F. Lund, Andrew W. Heath, Nanette Solvason, Wei Wei Wu, J. Christopher Grimaldi, R. M. E. Parkhouse and Maureen Howard. "CD38 unresponsiveness of xid B cells implicates Bruton's tyrosine kinase (btk) as a regulator of CD38 induced signal transduction." International Immunology, Vol 7(2), 163-170, 1995

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14. Nobuyuki Harada, Leopoldo Santos-Argumedo, Ray Chang, J. Christopher Grimaldi, Frances Lund, Camilynn I. Brannan, Neal G. Copeland, Nancy A. Jenkins, Andrew Heath, R. M. E. Parkhouse and Maureen Howard. "Expression Cloning of a cDNA Encoding a Novel Murine B Cell Activation Marker: Homology to Human CD38." The Journal of Immunology, Vol. 151, 3111-3118, 1993

15. David J. Rawlings, Douglas C. Saffran, Satoshi Tsukada, David A. Largaespada, J. Christopher Grimaldi, Lucie Cohen Randolph N. Mohr, J. Fernando Bazan, Maureen Howard, Neal G. Copeland, Nancy A. Jenkins, Owen Witte. "Mutation of Unique Region of Bruton's Tyrosine Kinase in Immunodeficient XID Mice." Science, Vol. 261, 358-360, 1993

16. J. Christopher Grimaldi, Raul Torres, Christine A. Kozak, Ray Chang, Edward Clark, Maureen Howard, and Debra A. Cockayne. "Genomic Structure and Chromosomal Mapping of the Murine CD40 Gene." The Journal of Immunology, Vol 149, 3921-3926, 1992

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Division into Peripheral Monoclonal, Polyclonal and Central Nervous System Lymphoma." AIDS, Vol. 5, 669-674, 1991

18. Ann Grimaldi and Chris Grimaldi. "Small-Scale Lambda DNA Prep." Contribution to Current Protocols in Molecular Biology, Supplement 5, Winter 1989

19. J. Christopher Grimaldi, Timothy C. Meeker. "The t(5;14) Chromosomal Translocation in a Case of Acute Lymphocytic Leukemia Joins the Interleukin-3 Gene to the Immunoglobulin Heavy Chain Gene." Blood, Vol. 73, 2081-2085, 1989

20. Timothy C. Meeker, J. Christopher Grimaldi, et al. "An Additional Breakpoint Region in the BCL-1 Locus Associated with the t(11;14) (q13;q32) Translocation of B-Lymphocytic Malignancy." Blood, Vol. 74, 1801-1806, 1989

21 Timothy C. Meeker, J. Christopher Grimaldi, Robert O'Rourke, et al. "Lack of Detectable Somatic Hypermutation in the V Region of the Ig H Chain Gene of a Human Chronic B Lymphocytic Leukemia." The Journal of Immunology, Vol. 141, 3994-3998, 1988

MANUSCRIPTS IN PREPARATION

1. Sriram Balasubramanian, J. Christopher Grimaldi, J. Fernando Bazan, Gerard Zurawski and Maureen Howard. "Structural and functional characterization of CD38: Identification of active site residues"

PATENTS

1. "Methods for Eosinophil Depletion with Antibody to CCR3 Receptor" (US 6,207,155 B1).

2. "Amplification Based Cloning Method." (US 6,607,899)

3. Ashkenazi et al., "Secreted and Transmembrane Polypeptides and Nucleic Acids Encoding the Same." (this patent covers several hundred genes)

4. "IL-17 Homologous Polypeptides and Therapeutic Uses Thereof"

5. "Method of Diagnosing and Treating Cartilaginous Disorders."

MEMBERSHIPS AND ACTIVITIES

Editor   Frontiers in Bioscience

Member   DNAX Safety Committee 1991-1999
                Biological Safety Affairs Forum (BSAF) 1990-1991
                Environmental Law Foundation (ELF) 1990-1991

The t(5;14) Chromosomal Translocation in a Case of Acute Lymphocytic Leukemia Joins the Interleukin-3 Gene to the Immunoglobulin Heavy Chain Gene

By J. Christopher Grimaldi and Timothy C. Meeker

Chromosomal translocations have proven to be important markers of the genetic abnormalities central to the pathogenesis of cancer. By cloning chromosomal breakpoints one can identify activated proto-oncogenes. We have studied a case of B-lineage acute lymphocytic leukemia (ALL) that was associated with peripheral blood eosinophilia. The chromosomal translocation t(5;14) (q31;q32) from this sample was cloned and studied at the molecular level. This translocation joined the immunoglobulin heavy chain joining (Jh) region to the promotor region of the interleukin-3 (IL-3) gene in opposite transcriptional orientations. The data suggest that activation of the IL-3 gene by the enhancer of the immunoglobulin heavy chain gene may play a central role in the pathogenesis of this leukemia and the associated eosinophilia.

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KARYOTYPIC STUDIES of leukemia and lymphoma have identified frequent nonrandom chromosomal translocations. Some of these translocations juxtapose the immunoglobulin heavy chain (IgH) gene with important
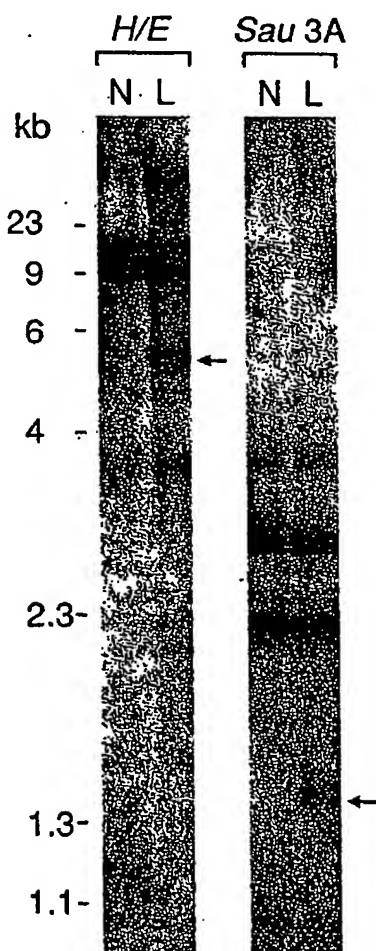

Fig 1. DNA blots of the leukemia sample. The restriction fragment pattern of normal human DNA (N) and the leukemia sample (L) were compared using a human Jh probe. Rearranged bands are indicated by arrows. Sample L exhibits a single rearranged band with both Hind III/EcoRI and Sau3A restriction digests. The rearranged bands are less intense than the other bands because the majority of cells in the sample represent normal bone marrow elements.

protooncogenes, such as c-myc and bcl-2.[1,2] In this way, the IgH gene can activate proto-oncogenes, resulting in disordered gene expression and a step in the development of cancer. The investigation of additional nonrandom translocations into the IgH locus allows us to identify new genes promoting the generation of leukemia and lymphoma.

A distinct subtype of acute lymphocytic leukemia (ALL) has been characterized by B-lineage phenotype, associated eosinophilia in the peripheral blood, and a t(5;14)(q31;q32) chromosomal translocation.[3,4] This syndrome probably occurs in <1% of all patients with ALL. We hypothesized that the cloning of the translocation characteristic of this leukemia might allow the identification of an important gene on chromosome 5 that plays a role in the evolution of this disease. In this report we demonstrate that the interleukin-3 gene (IL-3) and the IgH gene are joined by this translocation.

MATERIALS AND METHODS

Sample and DNA blots. A bone marrow aspirate from a representative patient with ALL (L1 morphology by French-American-British [FAB] criteria), peripheral eosinophilia (up to 20,000 per microliter with a normal value of <350 per microliter) and a t(5;14)(q31;q32) translocation was studied. Using published methods, genomic DNA was isolated and DNA blots were made.[5] Briefly, 10 μg of high molecular weight (mol wt) DNA were digested using an appropriate restriction enzyme and electrophoresed on a 0.8% agarose gel. The gel was stained with ethidium bromide, photographed, denatured, neutralized, and transferred to Hybond (Amersham, Arlington Heights, IL). After treatment of the filter with ultraviolet light, hybridization was performed. The filter was washed to a final stringency of 0.2% saturated sodium citrate (SSC) and 0.1% sodium lauryl sulfate (SDS) and exposed to film. The human Jb probe has been previously reported.[6]

Genomic library. The genomic library was made using pub-

lished methods.[5] Approximately 100 μg of high mol wt genomic DNA were partially digested with the Sau3A restriction enzyme. Fragments from 9 to 23 kilobases (kb) in size were isolated on a sucrose gradient and ligated into phage EMBL3A (Strategene, San Diego). Recombinant phage were packaged, plated, and screened as previously reported.[5]

DNA sequencing. Fragments for sequencing were cloned into M13 vectors and sequenced by the chain termination method using Sequenase (United States Biochemical, Cleveland).[7] All sequence data were derived from both strands.

RESULTS

We studied a bone marrow sample from a patient with ALL and associated peripheral eosinophilia. Karyotypic analysis showed the characteristic t(5;14)(q31;q32) translocation. These features define a distinctive subtype of ALL.[3,4] The leukemic cells were analyzed for cell surface phenotype by immunofluorecence. They were positive for B1 (CD20), B4 (CD19), cALLA (CD10), HLA-DR, and terminal deoxynucleotidyl transferase (Tdt), but negative for surface immunoglobulin. This phenotypic profile describes an immature cell from the B-lymphocytic lineage.[8]

The leukemia DNA was analyzed by Southern blotting for rearrangements of the IgH gene. Using a human immunoglobulin Jh probe, a single rearranged band was detected by EcoRI, HindIII, SstI, Sau3A, and EcoRI plus HindIII restriction digests, suggesting rearrangement of one allele (Fig 1). The immunoglobulin Jh region from the other allele was presumably either deleted or in the germline configuration.

We hypothesized that the t(5;14)(q31;q32) juxtaposed a growth-promoting gene on chromosome 5 with the immunoglobulin Jh region on chromosome 14. Therefore, a genomic library was made from the leukemic sample and screened with a Jh probe. Fifteen distinct positive clones were isolated and screened for the presence of the rearranged Sau3A fragment that was detected by DNA blotting. By this analysis, five clones appeared to represent the rearranged allele identified by DNA blots. One of these clones (clone no. 4) was chosen for further study and a detailed restriction map was generated. The EcoRI, HindIII/EcoRI, and SstI fragments from clone no. 4 that hybridized to the human Jh probe were also identical in size to the rearranged fragments from the leukemia sample, confirming that clone no. 4 represented the rearranged leukemic allele.

Phage clone no. 4 contained 3.7 kb of unknown origin joined to the IgH gene in the region of Jh4 (Fig 2). The IgH gene from Jh4 to the Cmu region appeared to be in germline configuration. Previously, the gene encoding hematopoietic growth factor IL-3 had been mapped to chromosome 5q31 so it was suspected that clone no. 4 might contain part of this gene.[9-12] When the restriction map of human IL-3 and clone no. 4 were compared, they were identical for more than 3 kb (Fig 2).

We confirmed the juxtaposition of the IL-3 gene and the IgH gene by nucleic acid sequencing of the subcloned BstBII/HpaI fragment (Fig 2). The sequence of this fragment showed no disruption of the protein coding region or the messenger RNA of the IL-3 gene. The break in the IL-3 gene occurred in the promotor region, 452 base pairs (bp) upstream of the transcriptional start site (position 64, Fig
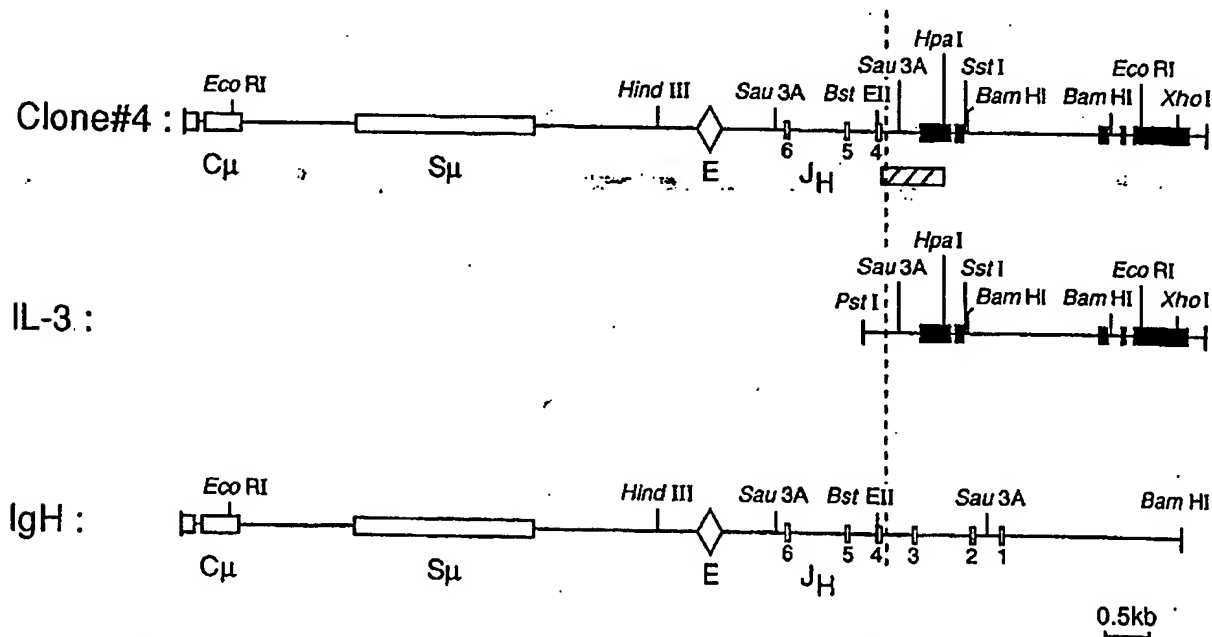
Fig 2. Breakpoint region: t(5;14)(q31;q32). Comparative mapping of phage clone no. 4, the germline IgJh region, and the germline IL-3 gene.[28,29] The map of clone no. 4 is identical to that of IgH until it diverges in the region of Jh4 (at the dashed line), after which it is identical to the map of IL-3. The two genes are positioned in a head-to-head orientation. The Ig μ chain constant region (Cμ), switch region (Sμ), enhancer (E), and Jh segments are indicated (open symbols). The five exons (dark boxes) and four introns of the IL-3 gene are shown. The hatched box indicates the sequenced region.

3A). The break in the IgH gene occurred 2 bp upstream of the Jh4 region. Between the two breaks, 25 bp of uncertain origin (putative N sequence) were inserted.[13,14] No sequences homologous to the immunoglobulin heptamer and nonamer could be identified in the IL-3 sequence (Fig 3B). Therefore, nucleic acid sequencing confirmed the juxtaposition of the IL-3 gene and the IgH gene. The sequence data clearly showed that the genes were positioned in opposite transcriptional orientations (head-to-head).

Available data also allowed us to determine the normal positions of the IL-3 gene and the GM-CSF gene in relation to the centromere of chromosome 5 (Fig 4). The IgH gene is known to be positioned with the variable regions toward the telomere on chromosome 14q.[2,15] It has also been shown that

GM-CSF maps within 9 kb of IL-3 in the same transcriptional orientation.[16] Using this information and assuming a simple translocation event in our sample, we can conclude that the IL-3 gene is normally more centromeric, and the GM-CSF gene.more telomeric on chromosome 5q (Fig 4). Furthermore, both are transcribed with their 5' ends toward the centromere.

DISCUSSION

In this report we have cloned a unique chromosomal translocation that appears to be a consistent feature of a rare, yet distinct, clinical form of acute leukemia. This translocation joined the promotor of the IL-3 gene to the IgH gene. Except for the altered promotor, the IL-3 gene appeared
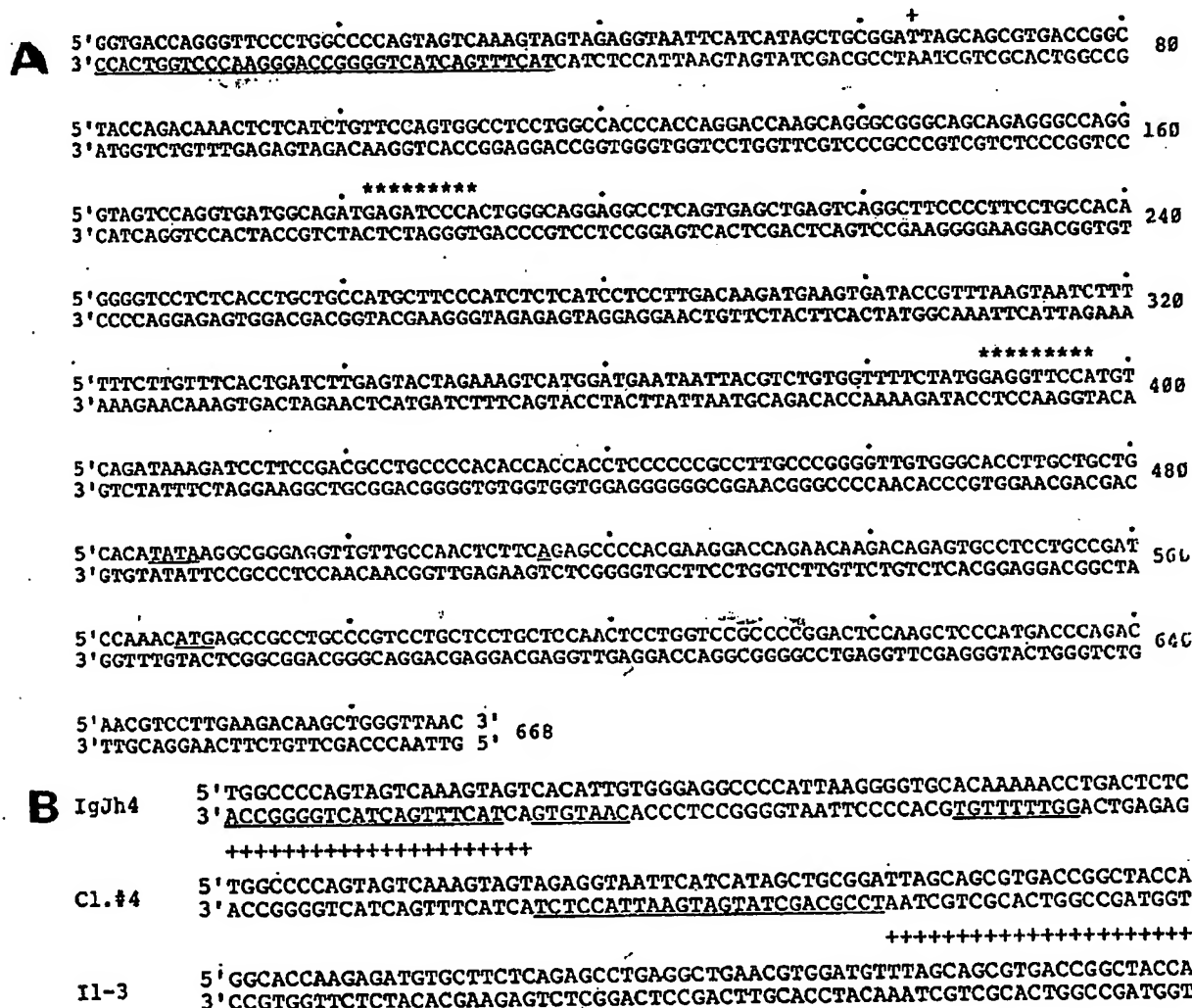
A

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5'GGTGACCAGGGTTCCCTGGCCCCAGTAGTCAAAGTAGTAGAGGTAATTCATCATAGCTGCGGATTAGCAGCGTGACCGGC  80
3'CCACTGGTCCCAAGGGACCGGGGTCATCAGTTTCATCATCTCCATTAAGTAGTATCGACGCCTAATCGTCGCACTGGCCG
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5'TACCAGACAAACTCTCATCTGTTCCAGTGGCCTCCTGGCCACCCACCAGGACCAAGCAGGGCGGGCAGCAGAGGGCCAGG  160
3'ATGGTCTGTTTGAGAGTAGACAAGGTCACCGGAGGACCGGTGGGTGGTCCTGGTTCGTCCCGCCCGTCGTCTCCCGGTCC
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5'GTAGTCCAGGTGATGGCAGATGAGATCCCACTGGGCAGGAGGCCTCAGTGAGCTGAGTCAGGCTTCCCCTTCCTGCCACA  240
3'CATCAGGTCCACTACCGTCTACTCTAGGGTGACCCGTCCTCCGGAGTCACTCGACTCAGTCCGAAGGGGAAGGACGGTGT
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5'GGGGTCCTCTCACCTGCTGCCATGCTTCCCATCTCTCATCCTCCTTGACAAGATGAAGTGATACCGTTTAAGTAATCTTT  320
3'CCCCAGGAGAGTGGACGACGGTACGAAGGGTAGAGAGTAGGAGGAACTGTTCTACTTCACTATGGCAAATTCATTAGAAA
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5'TTTCTTGTTTCACTGATCTTGAGTACTAGAAAGTCATGGATGAATAATTACGTCTGTGGTTTTCTATGGAGGTTCCATGT  400
3'AAAGAACAAAGTGACTAGAACTCATGATCTTTCAGTACCTACTTATTAATGCAGACACCAAAAGATACCTCCAAGGTACA
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5'CAGATAAAGATCCTTCCGACGCCTGCCCCACACCACCACCTCCCCCCGCCTTGCCCGGGGTTGTGGGCACCTTGCTGCTG  480
3'GTCTATTTCTAGGAAGGCTGCGGACGGGGTGTGGTGGTGGAGGGGGGCGGAACGGGCCCCAACACCCGTGGAACGACGAC
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5'CACATATAAGGCGGGAGGTTGTTGCCAACTCTTCAGAGCCCCACGAAGGACCAGAACAAGACAGAGTGCCTCCTGCCGAT  560
3'GTGTATATTCCGCCCTCCAACAACGGTTGAGAAGTCTCGGGGTGCTTCCTGGTCTTGTTCTGTCTCACGGAGGACGGCTA
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5'CCAAACATGAGCCGCCTGCCCGTCCTGCTCCTGCTCCAACTCCTGGTCCGCCCCGGACTCCAAGCTCCCATGACCCAGAC  640
3'GGTTTGTACTCGGCGGACGGGCAGGACGAGGACGAGGTTGAGGACCAGGCGGGGCCTGAGGTTCGAGGGTACTGGGTCTG
```

```
5'AACGTCCTTGAAGACAAGCTGGGTTAAC 3'  668
3'TTGCAGGAACTTCTGTTCGACCCAATTG 5'
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B

IgJh4
```
5'TGGCCCCAGTAGTCAAAGTAGTCACATTGTGGGAGGCCCCATTAAGGGGTGCACAAAAACCTGACTCTC
3'ACCGGGGTCATCAGTTTCATCAGTGTAACACCCTCCGGGGTAATTCCCCACGTGTTTTTGGACTGAGAG
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Cl.#4
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5'TGGCCCCAGTAGTCAAAGTAGTAGAGGTAATTCATCATAGCTGCGGATTAGCAGCGTGACCGGCTACCA
3'ACCGGGGTCATCAGTTTCATCATCTCCATTAAGTAGTATCGACGCCTAATCGTCGCACTGGCCGATGGT
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                           +++++++++++++++++++++++++
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Il-3
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5'GGCACCAAGAGATGTGCTTCTCAGAGCCTGAGGCTGAACGTGGATGTTTAGCAGCGTGACCGGCTACCA
3'CCGTGGTTCTCTACACGAAGAGTCTCGGACTCCGACTTGCACCTACAAATCGTCGCACTGGCCGATGGT
```

Fig 3. Sequence of t(5;14)(q31;q32) breakpoint region. (A) Nucleotide sequence of the BstEII/HpaI fragment indicated on Fig 2. Nucleotides 1 to 36 represent the Jh4 coding region underlined on the coding strand.[8] Nucleotides 39 to 63 are a putative N region. The sequence from position 64 to 668 is that of the germline IL-3 gene.[28] The IL-3 TATA box (485), transcription start (515), and initiation methionine (567) are underlined. Two proposed regulatory sequences in the promotor are marked by asterisks (positions 182 and 389). (B) Comparative sequence of the t(5;14)(q31;q32) breakpoint region. The IgJh4 region is shown with its coding region, heptamer, and nonamer underlined. Clone no. 4 is shown with putative N region sequences underlined. The IL-3 sequence is also shown. A plus sign (+) denotes the identical nucleotide between sequences. No heptamer or nonamer is identified in the IL-3 sequence.
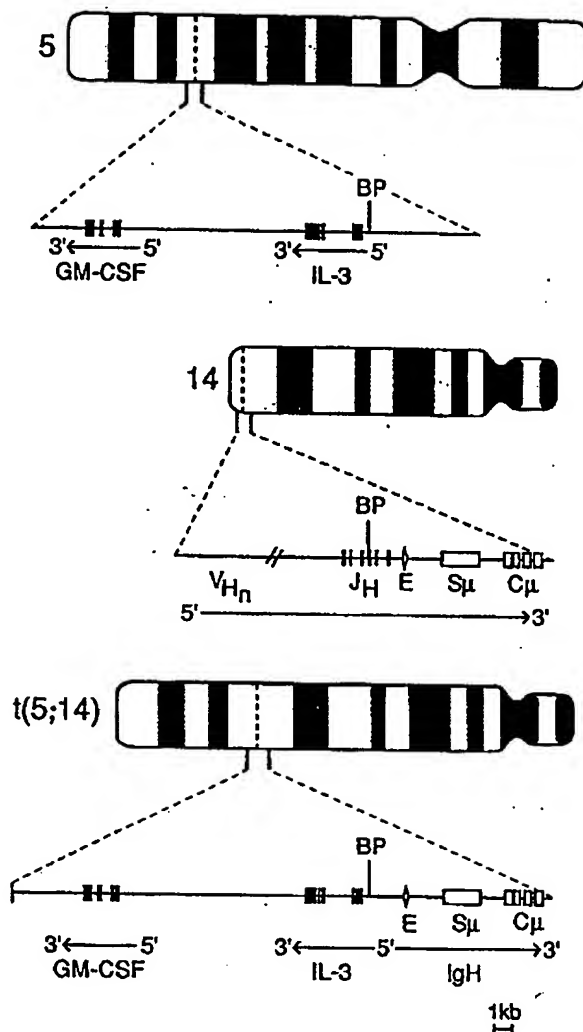
2084

GRIMALDI AND MEEKER

Fig 4. Diagram of the translocation. The normal chromosome 5q31 is shown with the GM-CSF gene telomeric to the IL-3 gene in the transcriptional orientation shown. On normal chromosome 14q32 the Vh regions are telomeric. The t(5;14)(q31;q32) translocation results in the head-to-head orientation of these genes. Symbols are defined in Fig 2. BP, breakpoint position.

intact as no deletions, insertions, or point mutations were detected by restriction mapping of the entire gene and sequencing of part of the gene. The IgH gene has been truncated at the Jh4 region, which places the immunoglobulin enhancer within 2.5 kb of the IL-3 gene.[17,18] This leads to the hypothesis that the enhancer is increasing transcription of a structurally normal IL-3 gene. The same mechanism is important for activation of the c-myc gene in some cases of Burkitt's lymphoma.[19] An alternate hypothesis is that the elimination of an upstream IL-3 promotor element is crucial to the activation of the IL-3 gene.

The proposed activation of the IL-3 gene suggests that an autocrine loop is important for the pathogenesis of this leukemia.[20] Over-expression of the IL-3 gene coupled with

the presence of the IL-3 receptor in these cells could account for a strong stimulus for proliferation. In this regard, there are data indicating that immature B-lineage lymphocytes and B-lineage leukemias may express the IL-3 receptor.[21,22]

An additional feature of this type of leukemia is the dramatic eosinophilia, consisting of mature forms. It has been hypothesized that the eosinophils do not arise from the malignant clone, but are stimulated by the tumor.[23,24] Because of the known effect of IL-3 on eosinophil differentiation, secretion of high levels of IL-3 by leukemic cells might have a role in the eosinophilia in this type of leukemia.[12]

The data suggest that the recombination mechanism that is active in the IgH gene during normal differentiation has a role in this translocation.[13,14] This is supported by the breakpoint location at the 5' end of Jh4 and the presence of putative N-region sequences. On the other hand, no recombination signal sequence (heptamer and nonamer) was found in this region on chromosome 5, suggesting that additional factors also played a role. Further studies will elucidate the mechanism of this and other translocations.

In the leukemia we studied, it is possible that the immunoglobulin enhancer also activates the GM-CSF gene, since this gene is probably positioned only 14 kb away (Fig 4). This is known to be within the range of enhancer activation.[25] The interleukin-5 (IL-5) gene maps to chromosome 5q31.[26] Deregulation of the IL-5 gene by this translocation would act synergistically with IL-3 in the stimulation of eosinophil proliferation and differentiation.[27] These and other questions will be answered by the study of more patient samples. We plan to determine whether the t(5;14)(q31;q32) translocation is capable of activating multiple lymphokines simultaneously and whether they cooperate in the generation of this leukemia.

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RAPID COMMUNICATION

Activation of the Interleukin-3 Gene by Chromosome Translocation in Acute Lymphocytic Leukemia With Eosinophilia

By Timothy C. Meeker, Dan Hardy, Cheryl Willman, Thomas Hogan, and John Abrams

The t(5;14)(q31:q32) translocation from B-lineage acute lymphocytic leukemia with eosinophilia has been cloned from two leukemia samples. In both cases, this translocation joined the IgH gene and the interleukin-3 (IL-3) gene. In one patient, excess IL-3 mRNA was produced by the leukemic cells. In the second patient, serum IL-3 levels were measured and shown to correlate with disease activity. There was no evidence of excess granulocyte/macrophage colony stimulating factor (GM-CSF) or IL-5 expression. Our data support the formulation that this subtype of leukemia may arise in part because of a chromosome translocation that activates the IL-3 gene, resulting in autocrine and paracrine growth effects.

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A NUMBER OF chromosome translocations have been associated with human leukemia and lymphoma. In many cases the study of these translocations has led to the discovery or characterization of proto-oncogenes, such as bcl-2, c-abl, and c-myc, that are located adjacent to the translocation.[1,2] It is now widely understood that cancer-associated translocations disrupt nearby proto-oncogenes.

A distinct subtype of acute leukemia is characterized by the triad of B-lineage immunophenotype, eosinophilia, and the t(5;14)(q31;q32) translocation.[3,4] Leukemic cells from such patients have been positive for terminal deoxynucleotidyl transferase (Tdt), common acute lymphoblastic leukemia antigen (CALLA), and CD19, but negative for surface or cytoplasmic immunoglobulin. In previous work, we cloned the t(5;14) breakpoint from one leukemic sample (Case 1) and determined that the IgH and interleukin-3 (IL-3) genes were joined by this abnormality.[5] In this report, we extend those findings by showing that the t(5;14)(q31;q32) translocation from a second leukemia sample (Case 2) has a similar structure, and we report our study of growth factor expression in these patients.

MATERIALS AND METHODS

Samples and Southern blots. Case 1 has been described.[5,6] Clinical features of Case 2 have been described in detail.[3] DNA isolation and Southern blotting was done using previously described methods.[5] Filters were hybridized with an immunoglobulin Jh probe, a 280 bp BamHI/EcoRI genomic IL-3 fragment, and an IL-3 cDNA probe.[7,8]

Northern blots. RNA isolation and Northern blotting have been described.[9] Briefly, Northern blots were done by separating 9μg total RNA on 1% agarose-formaldehyde gels. Equal RNA loading in each lane was confirmed by ethidium bromide staining. Blots were hybridized with an IL-3 cDNA probe extending to the Xho I site in exon 5, a 720 bp Sst I/Kpn I probe derived from intron 2 of the IL-3 gene, a 600 bp Nhe I/Hpa I IL-5 cDNA probe, and a 500 bp Pst I/Nco I granulocyte-macrophage colony stimulating factor (GM-CSF) cDNA probe.[10-12]

Polymerase chain reaction. Primers were designed with BamHI sites for cloning. One primer hybridized to the Jh sequences from the IgH gene (Primer 144:5'-TAGGATCCGACGGTGACCAGGGT), and the other hybridized to the region of the TATA box in the IL-3 gene (Primer 161: 5'-AACAGGATCCCGCCTTATATGTCAG). Polymerase chain reaction (PCR) (95°C for 1 minute, 61°C for 30 seconds, and 72°C for 3 minutes) was done using 500 ng genomic DNA and 50 pmol of each primer in 100 μL containing 67 mmol/L Tris-HCl pH 8.8, 6.7 mmol/L MgCl₂, 10% dimethyl sulfoxide (DMSO), 170 μg/mL bovine serum albumin (BSA) (fraction V),

16.6 mmol/L ammonium sulfate, 1.5 mmol/L each dNTP and Taq polymerase (Perkin-Elmer, Norwalk, CT).[13]

Sequencing. Sequencing was done by chain termination in M13 vectors.[14] As part of this study, we sequenced a subclone of a normal IL-3 promotor, covering 598 base pairs from a Sma I site at position −1240 (with respect to the proposed site of transcription initiation) to an Nhe I site at position −642. The plasmid containing this region was a gift from Naoko Arai of the DNAX Research Institute.

Expression in Cos7 cells. A genomic IL-3 fragment from Case 1 was cloned into the pXM expression vector.[10] Briefly, the HindIII/Sal I fragment containing the IL-3 gene was subcloned from the previously described phage clone 4 into pUC18.[5] The 2.6 kb fragment extending from the Sma I site 61 bp upstream of the IL-3 transcription start to the Sma I site in the polylinker was cloned into the blunted Xho I site of pXM. The negative control construct was the pXM vector without insert. Plasmids were introduced into Cos7 cells by electroporation, and supernatant was collected after 48 hours in culture.

TF1 bioassay. TF-1 cells were passaged in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol L-glutamine, and 1 ng/mL human GM-CSF.[15] Samples and antibodies were diluted in this same medium lacking GM-CSF but containing penicillin and streptomycin. A 25 μL volume of serial dilutions of patient serum was added to wells in a flat bottom 96-well microtiter plate. Rat anti-cytokine monoclonal antibody in a volume of 25 μL was added to appropriate wells and preincubated for 1 hour at 37°C. Fifty microliters of twice washed TF-1 cells were added to each well, giving a final cell concentration of 1 × 10⁴ cells per well. (final volume, 100 μL). The plate was incubated for 48 hours. The remaining cell viability was determined metabolically by the colori-

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IgJh5   5'GACGGTGACCAGGGTTCCTTGGCCCCAGGAGTCGAACCAGTTGTCACATTGTGACAACAATGCCAGACCCCGACAAAGAACGGGCGCCCCGCACGTCCCT
        3'CTGCCACTGGTCCCAAGGGAACCGGGGTCCTCAGCTTGGTCAACAGTGTAACACTGTTGTTACGGTCTGGGGCTGTTTCTTGCCCGCGGGGCGTGCAGGGA

        +++++++++++++++++++ ++++++++++ ++++++++++++++

Clone   5'GAGGGTGACCAGGGTTCCCTGGCCCCAGGGGTCGAACCAGTTGTACCGCCATGTTATTGGGGGGGATCAAGACCTCTCAATACAACTGTCTCCTGCCAAT
        3'CTGCCACTGGTCCCAAGGGACCGGGGTCCCCAGCTTGGTCAACATGGCGGTACAATAACCCCCCCTAGTTCTGGAGAGTTATGTTGACAGAGGACGGTTA

                                                (-934)*+++++++++++++++++++++++++++++++++++++++++++++++++

IL3     5'CCCCTCTCTGCAAACCTTGCCTACTGGGCCTGCACCTGGCAAATCCATGCTCAGCACCAGACGGGGATCAAGACCTCTCAATACAACTGTCTCCTGCCAAT
        3'GGGGAGAGACGTTTGGAACGGATGACCCGGACGTGGACCGTTTAGGTACGAGTCGTGTCTGCCCCTAGTTCTGGAGAGTTATGTTGACAGAGGACGGTTA
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Fig 1. Breakpoint sequences for Case 2. The germline IgJh5 region sequence (protein coding region and recombination signal sequences are underlined) is on top, the translocation sequence from Case 2 (PCR primer sequences and putative N region are underlined) is in the middle, and the germline IL-3 sequence, which we derived from a normal IL-3 clone, is on the bottom.[7] + indicates that each sequence has the same nucleotide. The sequence documents the head-to-head joining of the IL-3 and IgH genes. The breakpoint in the IL-3 gene occurred at position −934 (*).

metric method of Mosmann using a VMax microtiter plate reader (Molecular Devices, Menlo Park, CA) set at 570 and 650 nm.[16]

Cytokine immunoassays. These assays used rat monoclonal anti-cytokine antibodies (10 μg/mL) to coat the wells of a PVC microtiter plate. The capture antibodies used were BVD3-6G8, JES1-39D10, and BVD2-23B6, for the IL-3, IL-5, and GM-CSF assays, respectively. Patient sera were then added (undiluted and diluted 1:2 for IL-3, undiluted for IL-5, and undiluted and diluted 1:5 for GM-CSF). The detecting immunoreagents used were either mouse antiserum to IL-3 or nitroiodophenyl (NIP)-derivatized rat monoclonal antibodies JES1-5A2 and BVD2-21C11, specific for IL-5 and GM-CSF, respectively. Bound antibody was subsequently detected with immunoperoxidase conjugates: horseradish peroxidase (HRP)-labeled goat anti-mouse Ig for IL-3, or HRP-labeled rat (J4 MoAb) anti-NIP for IL-5 and GM-CSF. The chromogenic substrate was 3-3'azino-bis-benzthiazoline sulfonate (ABTS; Sigma, St Louis, MO). Unknown values were interpolated from standard curves prepared from dilutions of the recombinant factors using Softmax software available with the VMAX microplate reader (Molecular Devices).

RESULTS

Leukemic DNA from Case 2 was studied by Southern blotting. When digested with the HindIII restriction enzyme and hybridized with a human immunoglobulin heavy chain joining region (Jh) probe, a rearranged fragment at approximately 14 kb was detected (data not shown). When reprobed with either of two different IL-3 probes, a rearranged 14 kb

fragment, comigrating with the rearranged Jh fragment, was identified. When leukemic DNA was digested with HindIII plus EcoRI, a rearranged Jh fragment was detected at 6 kb. The IL-3 probes also identified a comigrating fragment of this size. These experiments indicated that the leukemic sample studied was clonal and that a single fragment contained both Jh and IL-3 sequences, suggesting a translocation had occurred.

To characterize better the joining of the IL-3 gene and the immunoglobulin heavy chain (IgH) gene, the polymerase chain reaction (PCR) was used to clone the translocation.[13] A Jh primer and an IL-3 primer were designed to produce an amplified product in the event of a head-to-head translocation. While control DNA gave no PCR product, Case 2 DNA yielded a PCR-derived fragment of approximately 980 bp, which was cloned and sequenced.

The DNA sequence of the translocation clone from Case 2 confirmed the joining of the Jh region with the promotor of the IL-3 gene in a head-to-head configuration (Fig 1). Sequence analysis indicated that the breakpoint on chromosome 14 was just upstream of the Jh5 coding region. The breakpoint on chromosome 5 occurred 934 bp upstream of the putative site of transcription initiation of the IL-3 gene. We also determined that a putative N sequence of 17 bp was inserted between the chromosome 5 and chromosome 14 sequences during the translocation event.[17,18] Figure 2 shows

0.5 Kb
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Fig 2. Relationship of chromosome 5 breakpoints to the IL-3 gene. This figure shows the two cloned breakpoints (arrows) in relation to the normal IL-3 gene.[9,10] One breakpoint occurred at position −462 and the other at −934 (arrows). In both circumstances, the translocations resulted in a head-to-head joining of the IgH gene and the IL-3 gene, leaving the mRNA and protein coding regions of the IL-3 gene intact. Boxes denote the five IL-3 exons; restriction enzymes are (B) BamHI, (P) Pst I, (H) Hpa I, (E) EcoRI, and (X) Xho I.

Fig 3. Documentation of IL-3 mRNA over-expression. A Northern blot was prepared and hybridized with a probe for IL-3. Lane 1 contained RNA from unstimulated peripheral blood lymphocytes (PBL) as a negative control. Lane 2 contained RNA from PBL stimulated for 4 hours with concanavalian A (ConA), and lane 3 contained RNA from PBL stimulated with ConA for 48 hours. As in the positive control lanes (2 and 3), a 1 kb band was identified in the leukemic sample from Case 1 (lane 4, lower arrow), suggesting aberrant expression of the IL-3 gene. In addition, the leukemic sample showed over-expression of an unspliced 2.9 kb IL-3 transcript (lane 4, upper arrow). We documented that this represented an unspliced precursor of the mature 1 kb transcript by showing that this band hybridized to a probe from intron 2 of the IL-3 gene. A similar 2.9 kb band was detected in lane 2, suggesting that an IL-3 mRNA of this size is sometimes detectable in normal mitogen-stimulated cells. Lane 5 through 10 represent RNA from six samples of B-lineage acute lymphocytic leukemia without the t(5;14) translocation, indicating that only the sample with the translocation exhibited IL-3 over-expression. Case 2 could not be analyzed by Northern blot because too few cells were available for study.

the locations of the two cloned breakpoints in relation to the IL-3 gene. The two chromosome 5 breakpoints were separated by less than 500 bp.

The genomic structure in Cases 1 and 2 suggested that a normal IL-3 gene product was over-expressed as a result of the altered promotor structure. This would predict that the IL-3 gene on the translocated chromosome was capable of making IL-3 protein. This prediction was tested by expressing a genomic fragment from the translocated allele of Case 1 containing all five IL-3 exons under the control of the SV40 promotor/enhancer in the Cos7 cell line. Cell supernatants were studied in a proliferation assay using the factor dependent erythroleukemic cell line, TF-1. The supernatants derived from transfections using the vector plus insert supported TF-1 proliferation, while supernatants from transfections using the vector alone were negative in this assay (data not shown). Furthermore, the biologic activity could be blocked by an antibody to human IL-3 (BVD3-6G8). This result showed that the translocated allele retained the ability to make IL-3 mRNA and protein.

The level of expression of IL-3 mRNA in leukemic cells from Case 1 was assessed. Northern blotting showed that the mature IL-3 mRNA (approximately 1 kb) and a 2.9 kb unspliced IL-3 mRNA were excessively produced by the leukemia (Fig 3). The 2.9 kb form of the mRNA is also present at low levels in normal peripheral blood T lymphocytes after mitogen activation (Fig 3). Several B-lineage acute leukemia samples without the t(5;14) translocation had undetectable levels of IL-3 mRNA in these experiments. In addition, although genes for GM-CSF and IL-5 map close to the IL-3 gene and might have been deregulated by the translocation, no IL-5 or GM-CSF mRNA could be detected in the leukemic sample (data not shown).[19,20]

Three serum samples from Case 2 were assayed by immunoassay for levels of IL-3, GM-CSF, and IL-5 (Table 1). Serum IL-3 could be detected and correlated with the clinical course. When the patient's leukemic cell burden was

highest, the IL-3 level was highest. No serum GM-CSF or IL-5 could be detected.

Since the IL-3 immunoassay measured only immunoreactive factor, we confirmed that biologically active IL-3 was present by using the TF-1 bioassay. This bioassay can be rendered monospecific using appropriate neutralizing monoclonal antibodies specific for IL-3, IL-5, or GM-CSF. We observed that sera from 1-16-84 and 3-14-84 contained TF-1 stimulating activity that could be blocked with anti-IL-3 MoAb (BVD3-6G8), but not with MoAbs to IL-5 (JES1-39D10) or GM-CSF (BVD2-23B6) (Fig 4; GM-CSF data not shown). The amount of neutralizable bioactivity in these two samples correlated very well with the difference in IL-3 levels obtained by immunoassay for these samples. Furthermore, the failure to block TF-1 proliferating activity with either anti-IL-5 or anti-GM-CSF was consistent with the inability to measure these factors by immunoassay and

Table 1. Peripheral Blood Counts and Growth Factor Levels at Different Times in Case 2

	Sample Date		
	11/15/83	1/16/84	3/14/84
Peripheral blood counts (cells/μL)			
WBC	81,800	116,500	12,300
Lymphoblasts	0	33,785	0
Eosinophils	48,626	73,080	615
Serum growth factor levels (pg/mL)			
IL-3	<444	7,995	1,051
GM-CSF	<15	<15	<15
IL-5	<50	<50	<50

Peripheral blood counts from Case 2 at three different time points with the corresponding growth factor levels quantified by immunoassay. The patient received chemotherapy between 1/16/84 and 3/14/84 to lower his leukemic burden.[3] No serum samples were available for a similar analysis of Case 1.

Abbreviation: WBC, white blood cells.
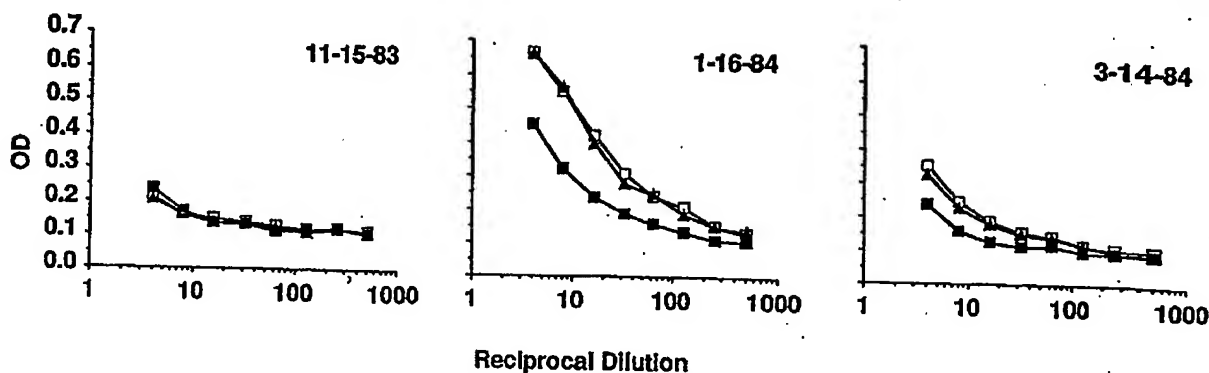
Fig 4. Bioassay of serum IL-3. Leukemic patient sera were tested for bioactive IL-3 and IL-5 in the TF-1 proliferation assay. The reciprocal of the dilution is indicated on the horizontal axis and the optical density indicating the amount of proliferation is indicated on the vertical axis. Serum from all three time points was assayed simultaneously. The assay was rendered monospecific by using a 1 µg/mL final concentration of monoclonal rat anti–IL-3, BVD3-6G8 (■), or anti–IL-5, JES1-39D10 (▲); □ indicates no MoAb. On 1/16/84 and 3/14/84, inhibition of proliferation was evident in the presence of anti–IL-3 antibody, documenting serum levels of IL-3 on those days. Serum IL-5 was not detected in this assay, as anti–IL-5 did not alter TF-1 proliferation.

indicated that these other myeloid growth factors were not detectably circulating in the serum of this patient.

DISCUSSION

In this report, we have extended our analysis of acute lymphocytic leukemia and eosinophilia associated with the t(5;14) translocation. In both cases we have studied, we have documented the joining of the IL-3 gene from chromosome 5 to the IgH gene from chromosome 14. The breakpoints on chromosome 5 are within 500 bp of each other, suggesting that additional breakpoints will be clustered in a small region of the IL-3 promotor. The PCR assay we have developed will be useful in the screening of additional clinical samples for this abnormality.

The finding of a disrupted IL-3 promotor associated with an otherwise normal IL-3 gene implied that this translocation might lead to the over-expression of a normal IL-3 gene product. In this work, we have documented that this is true. In addition, neither GM-CSF nor IL-5 are over-expressed by the leukemic cells. Furthermore, in one patient, serum IL-3 could be measured and correlated with disease activity. To our knowledge, this is the first measurement of human IL-3 in serum and its association with a disease process. The measurement of serum IL-3 in this and other clinical settings may now be indicated.

The finding of the IL-3 gene adjacent to a cancer-associated translocation breakpoint suggests that its activation is important for oncogenesis. It is our thesis that an autocrine loop for IL-3 is important for the evolution of this leukemia.[21] The excessive IL-3 production that we have documented would be one feature of such an autocrine loop. The final proof of our thesis must await additional data. In particular, from the study of additional clinical samples, it will be necessary to document that the IL-3 receptor is present on the leukemic cells and that anti–IL-3 antibody decreases proliferation of the leukemia in vitro.

An important aspect of this work is the suggestion of a therapeutic approach for this disease. If an autocrine loop for IL-3 can be documented in this disease, attempts to lower circulating IL-3 levels or block the interaction of IL-3 with its receptor may prove useful. Because it is also possible that the eosinophilia in these patients is mediated by the paracrine effects of leukemia-derived IL-3, similar interventions may improve this aspect of the disease. Antibodies or engineered ligands to accomplish these goals may soon be available.

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Clinical and Pathologic Significance of the c-erbB-2 (HER-2/neu) Oncogene

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The c-erbB-2 oncogene was first shown to have clinical significance in 1987 by Slamon et al,[79] who reported that c-erbB-2 DNA amplification in breast carcinomas correlated with decreased survival in patients with metastasis to axillary lymph nodes. Subsequent studies, however, of c-erbB-2 activation in breast carcinoma reached conflicting conclusions about its clinical significance. This oncogene also has been reported to have clinical and pathologic implications in other neoplasms. Our review summarizes these various studies and examines the clinical relevance of c-erbB-2 activation, which has not been emphasized in recent reviews.[37,38,55] The molecular biology of the c-erbB-2 oncogene has been extensively reviewed[37,38,55] and will be discussed only briefly here.

BACKGROUND

The c-erbB-2 oncogene was discovered in the 1980s by three lines of investigation. The *neu* oncogene was detected as a mutated transforming gene in neuroblastomas induced by ethylnitrosurea treatment of fetal rats.[8,73,74,78] The c-erbB-2 was a human gene discovered by its homology to the retroviral gene v-erbB.[33,49,76] HER-2 was isolated by screening a human genomic DNA library for homology with v-erbB.[24] When the DNA sequences were determined subsequently, c-erbB-2, HER-2, and *neu* were found to represent the same gene. Recently, the c-erbB-2 oncogene also has been referred to as *NGL*.

The c-erbB-2 DNA is located on human chromosome 17q21[24,33,68] and codes for c-erbB-2 mRNA (4.6 kb), which translates c-erbB-2 protein (p185). This

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protein is a normal component of cytoplasmic membranes. The c-erbB-2 oncogene is homologous with, but not identical to, c-erbB-1, which is located on chromosome 7 and codes for the epidermal growth factor receptor.[8,103] The c-erbB-2 protein is a receptor on cell membranes and has intracellular tyrosine kinase activity and an extracellular binding domain.[2,105] Electron microscopy with a polyclonal antibody detects c-erbB-2 immunoreactivity on cytoplasmic membranes of neoplasms, especially on microvilli and the non-villous outer cell membrane.[61] In normal cells, immunohistochemical reactivity for c-erbB-2 is frequently present at the basolateral membrane or the cytoplasmic membrane's brush border.[22,62]

There is experimental evidence that c-erbB-2 protein may be involved in the pathogenesis of breast neoplasia. Overproduction of otherwise normal c-erbB-2 protein can transform a cell line into a malignant phenotype.[25] Also, when the neu oncogene containing an activating point mutation is placed in transgenic mice with a strong promoter for increased expression, the mice develop multiple independent mammary adenocarcinomas.[16,63] In other experiments, monoclonal antibodies against the neu protein inhibit the growth (in nude mice) of a neu-transformed cell line,[26-28] and immunization of mice with neu protein protects them from subsequent tumor challenge with the neu-transformed cell line.[14] Some authors have speculated that the use of antagonists for the unknown ligand could be useful in future chemotherapy.[85] Further review of this experimental evidence is beyond the scope of this article.

The c-erbB-2 activation most likely occurs at an early stage of neoplastic development. This hypothesis is supported by the presence of c-erbB-2 activation in both in situ and invasive breast carcinomas. In addition, studies of metastatic breast carcinomas usually demonstrate uniform c-erbB-2 activation at multiple sites in the same patient,[11,12,39,41,62] although c-erbB-2 activation has rarely been detected in metastatic lesions but not in the primary tumor.[57,60,107] Even more rarely, c-erbB-2 DNA amplification has been detected in a primary breast carcinoma but not in its lymph node metastasis.[5] In patients who have bilateral breast neoplasms, both lesions have similar patterns of c-erbB-2 activation, but only a few such cases have been studied.[11]

MECHANISMS OF c-erbB-2 ACTIVATION

The most common mechanism of c-erbB-2 activation is genomic DNA amplification, which almost always results in overproduction of c-erbB-2 mRNA and protein.[17,34,65,81] The c-erbB-2 amplification may stabilize the overproduction of mRNA or protein through unknown mechanisms. Human breast carcinomas with c-erbB-2 amplification contain 2 to 40 times more c-erbB-2 DNA[43] and 4 to 128 times more c-erbB-2 mRNA[34,90] than found in normal tissue. Most human breast carcinomas with c-erbB-2 amplification have 2 to 15 times more c-erbB-2 DNA. Tumors with greater amplification tend to have greater overproduction.[17,52,65] The non-mammary neoplasms that have been studied tend to have

similar levels of c-erbB-2 amplification or overproduction relative to the corresponding normal tissue.

The second most common mechanism of c-erbB-2 activation is overproduction of c-erbB-2 mRNA and protein without amplification of c-erbB-2 DNA.[81] The quantities of mRNA and protein usually are less than those in amplified cases and may approach the small quantities present in normal breast or other tissues.[17,50,53] The c-erbB-2 protein overproduction without mRNA overproduction or DNA amplification has been described in a few human breast carcinoma cell lines.[47]

Other rare mechanisms of c-erbB-2 activation have been reported. Translocations involving the c-erbB-2 gene have been described in a few mammary and gastric carcinomas, although some reported cases may represent restriction fragment length polymorphisms or incomplete restriction enzyme digestions that mimic translocations.[31,65,75,84,90,108] A single point mutation in the transmembrane portion of neu has been described in rat neuroblastomas induced by ethylnitrosurea.[9,55] The mutated neu protein has increased tyrosine kinase activity and aggregates at the cell membrane.[10,83,98] Although there has been speculation that some of the amplified c-erbB-2 genes may contain point mutations,[46] none has been detected in primary human neoplasms.[41,53,81]

TECHNIQUES FOR DETECTING c-erbB-2 ACTIVATION

Detection of c-erbB-2 DNA Amplification

Amplification of c-erbB-2 DNA is usually detected by DNA dot blot or Southern blot hybridization. In the dot blot method, the extracted DNA is placed directly on a nylon membrane and hybridized with a c-erbB-2 DNA probe. In the Southern blot method, the extracted DNA is treated with a restriction enzyme, and the fragments are separated by electrophoresis, transferred to a nylon membrane, and hybridized with a c-erbB-2 DNA probe. In both techniques, c-erbB-2 amplification is quantified by comparing the intensity (measured by densitometry) of the hybridization bands from the sample with those from control tissue.

Several technical problems may complicate the measurement of c-erbB-2 DNA amplification. First, the extracted tumor DNA may be excessively degraded or diluted by DNA from stromal cells.[81] Second, the c-erbB-2 DNA probe must be carefully chosen and labeled. For example, oligonucleotide c-erbB-2 probes may not be sensitive enough for measuring a low level of c-erbB-2 amplification, because diploid copy numbers can be difficult to detect (unpublished data). Third, the total amounts of DNA in the sample and control tissue must be compensated for, often with a probe to an unamplified gene. Many studies have used control probes to genes on chromosome 17, the location of c-erbB-2, to correct for possible alterations in chromosome number. Identical results, however, are obtained by using control probes to genes on other chromosomes,[5,65,80] with rare exception.[17] Studies using control probes to the beta-

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globin gene must be interpreted with caution, because one allele of this gene is deleted occasionally in breast carcinomas.[3]

Amplification of c-erbB-2 DNA was assessed by using the polymerase chain reaction (PCR) in one recent study.[32] Oligoprimers for the c-erbB-2 gene and a control gene are added to the sample's DNA, and PCR is performed. If the sample contains more copies of c-erbB-2 DNA than of the control gene, the c-erbB-2 DNA is replicated preferentially.

Detection of c-erbB-2 mRNA Overproduction

Overproduction of c-erbB-2 mRNA usually is measured by RNA dot blot or Northern blot hybridization. Both techniques require extraction of RNA but otherwise are analogous to DNA dot blot and Southern blot hybridization. Use of PCR for detection of c-erbB-2 mRNA has been described in two recent abstracts.[68,102]

Overproduction of c-erbB-2 mRNA can be measured by in situ hybridization. Sections are mounted on glass slides, treated with protease, hybridized with a radiolabeled probe, washed, treated with nuclease to remove unbound probe, and developed for autoradiography. Silver grains are seen only over tumor cells that overproduce c-erbB-2 mRNA. Negative control probes are used.[65,96,106] Our experience indicates that these techniques are relatively insensitive for detecting c-erbB-2 mRNA overproduction in routinely processed tissue. Although the sensitivity may be increased by modifications that allow simultaneous detection of c-erbB-2 DNA and mRNA, in situ hybridization still is cumbersome and expensive (unpublished data).

All of the above c-erbB-2 mRNA detection techniques have several problems that make them more difficult to perform than techniques for detecting DNA amplification. One major problem is the rapid degradation of RNA in tissue that is not immediately frozen or fixed. In addition, during the detection procedure, RNA can be degraded by RNase, a ubiquitous enzyme, which must be eliminated meticulously from laboratory solutions. Third, control probes to genes that are uniformly expressed in the tissue of interest need to be carefully selected.

Detection of c-erbB-2 Protein Overproduction

The most accurate methods for detecting c-erbB-2 protein overproduction are the Western blot method and immunoprecipitation. Both techniques can document the binding specificity of various antibodies against c-erbB-2 protein. In Western blot studies, protein is extracted from the tissue, separated by electrophoresis (according to size), transferred to a membrane, and detected by using antibodies to c-erbB-2. In immunoprecipitation studies, antibodies against c-erbB-2 are added to a tumor lysate, and the resulting protein–antibody precipitate is separated by gel electrophoresis and stained for protein. Both Western blot and immunoprecipitation are useful research tools but currently are not practical for diagnostic pathology. Two recent abstracts have described an enzyme-linked immunosorbent assay (ELISA) for detection of c-erbB-2 protein.[19,45]

Overproduction of c-erbB-2 protein is most commonly assessed by various immunohistochemical techniques. These procedures often generate conflicting results, which are explained at least partially by three factors. First, various studies have used different polyclonal and monoclonal antibodies. Because some polyclonal antibodies recognize weak bands in addition to the c-erbB-2 protein band on Western blot or immunoprecipitation, the results of these studies should be interpreted with caution.[22,35,47,61] Even some monoclonal antibodies immunoprecipitate protein bands in addition to c-erbB-2 (p185).[30,59,88] Second, tissue fixation contributes to variability between studies. For example, some antibodies detect c-erbB-2 protein only in frozen tissue and do not react in fixed tissue. In general, formalin fixation diminishes the sensitivity of immunohistochemical methods and decreases the number of reactive cells.[61,85] When Bouin's fixative is used, there may be a higher percentage of positive cases.[22] Third, minimal criteria for interpreting immunohistochemical staining are generally lacking. Although there is general agreement that distinct crisp cytoplasmic membrane staining is diagnostic for c-erbB-2 activation in breast carcinoma, the number of positive cells and the staining intensity required to diagnose c-erbB-2 protein overproduction varies from study to study and from antibody to antibody. Degradation of c-erbB-2 protein is not a problem because it can be detected in intact form more than 24 hours after tumor resection without fixation or freezing.[64]

ACTIVATION OF c-erbB-2 IN BREAST LESIONS

Incidence of c-erbB-2 Activation

Most studies of c-erbB-2 oncogene activation do not specify histological subtypes of infiltrating breast carcinoma. Amplification of c-erbB-2 DNA was found in 19.1 percent (519 of 2715) of invasive carcinomas in 25 studies (Table 1), and c-erbB-2 mRNA or protein overproduction was detected in 20.9 percent (566 of 2714) of invasive carcinomas in 20 studies. Twelve studies have documented c-erbB-2 mRNA or protein overproduction in 15 percent (88 of 604) of carcinomas that lacked c-erbB-2 DNA amplification.

The incidence of c-erbB-2 activation in infiltrating breast carcinoma varies with the histological subtype. Approximately 22 percent (142 of 650) of infiltrating ductal carcinomas have c-erbB-2 activation, as expected from the above data. Other variants of breast carcinoma with frequent c-erbB-2 activation are inflammatory carcinoma (62 percent, 54 of 87), Paget's disease (82 percent, 9 of 11), and medullary carcinoma (22 percent, 5 of 23). In contrast, c-erbB-2 activation is infrequent in infiltrating lobular carcinoma (7 percent, 5 of 73) and tubular carcinoma (7 percent, 1 of 15).

The c-erbB-2 protein overproduction is present in 44 percent (44 of 100) of ductal carcinomas in situ and especially comedocarcinoma in situ (68 percent, 49 of 72). The micropapillary type of ductal carcinoma in situ also tends to have c-erbB-2 activation,[40,54,68] especially if larger cells are present. The greater fre-

TABLE 1. c-erbB-2 ACTIVATION IN MALIGNANT HUMAN BREAST NEOPLASMS

Histological Diagnosis	c-erbB-2 DNA Amplification[a]	c-erbB-2 mRNA Overproduction	c-erbB-2 Protein Overproduction[b]
Carcinoma, not otherwise specified	146/528,[31] 52/310,[17] 52/291,[100] 28/176,[67] 17/157,[113] 22/141,[35] 14/136,[57] 12/122,[1] 19/103,[79] 15/95,[90] 15/88,[111] 17/73,[7] 16/66,[42] 6/61,[80] 11/57,[92] 10/57,[65] 13/51,[113] 8/49,[91] 10/38,[92] 12/38,[94] 1/25,[15] 7/24,[81] 7/15,[91] 7/10,[98] 2/10[107]	42/180,[59] 49/126,[35] 19/62,[85] 19/57,[60] 3/11,[90] 8/10,[98] 3/8[91]	118/728,[80b] 58/330,[17b] 47/313,[66] 17/195,[113] 32/191,[88] 31/185,[101] 34/102,[42] 24/53,[82b] 23/47,[13] 22/45,[8] 11/36,[94] 7/24,[91] 1/10[81]
Carcinoma, type not specified but lacking c-erbB-2 DNA amplification	—	18/136,[81] 14/73,[34] 8/16,[69] 0/8,[90] 1/4,[91] 0/3[98]	16/231,[17b] 18/138,[81] 13/35,[13] 14/29,[80b] 1/28,[82] 3/24,[34] 0/17[91]
Infiltrating ductal carcinoma	21/118,[92] 23/107,[34] 17/50,[44] 7/37[90] 14/53 (comedo-carcinoma)[16] 3/33 (tubuloductal carcinoma)[16]	35/85[34]	22/137,[49] 14/93,[69] 9/34[88]

Inflammatory carcinoma	33/80,[35] 3/6[52]	46/75[35]	5/6[62]
Paget's disease	0/5,[18] 0/1[53]	—	5/6,[40] 2/3,[54] 2/2[62]
Tubular carcinoma		—	1/8[40]
Medullary carcinoma	2/4,[18] 0/1[54]	0/1[54]	1/12,[40] 1/3,[54] 1/2,[62] 0/1[39]
Mucinous carcinoma	0/1,[18] 0/1[62]		1/2[62]
Invasive papillary carcinoma	0/2[62]	—	
Infiltrating lobular carcinoma	1/15,[18] 0/6[54]	1/5[54]	2/27,[62] 0/12,[40] 0/9,[39] 1/5[62]
Mammary fibrosarcoma	0/1[53]		0/1[53]
"Benign cystosarcoma"	3/5[52]	?[?]	
Ductal CIS[c] with minimal invasion		—	33/74,[40] 10/24[39]
Ductal CIS	0/2[54]	1/2[54]	20/33,[34] 19/29,[62]
Ductal CIS, solid or comedo type		—	10/10[54]
Ductal CIS, micropapillary type		—	10/10[53]
Ductal CIS, micropapillary or cribriform type		—	1(focal)/1,[54]
Ductal CIS, papillary or cribriform type		—	0/16,[62] 1/9,[68] 0/3[40]
Lobular CIS		—	0/16[60]

[a] Shown as number of cases with activation/number of cases studied; reference is given as a superscript.

[b] These protein studies used Western blots; the rest used immunohistochemical methods.

[c] CIS = carcinoma in situ.

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quency of c-erbB-2 protein overproduction in comedocarcinoma in situ, compared with infiltrating ductal carcinoma, could be explained by the fact that many infiltrating ductal carcinomas arise from other types of intraductal carcinoma, which show c-erbB-2 activation infrequently. Others have speculated that carcinoma in situ with c-erbB-2 activation tends to regress or to lose c-erbB-2 activation during progression to invasion.[40,68,92] Infiltrating and in situ components of ductal carcinoma, however, usually are similar with respect to c-erbB-2 activation,[11,39] although some authors have noted more heterogeneity of the immunohistochemical staining pattern in invasive than in in situ carcinoma.[40,42,88] Activation of c-erbB-2 is infrequent in lobular carcinoma in situ. If lesions contain more than one histological pattern of carcinoma in situ, the c-erbB-2 protein overproduction tends to occur in the comedocarcinoma in situ but may include other areas of carcinoma in situ.[42,54,68] Overproduction of c-erbB-2 protein in ductal carcinoma in situ correlates with larger cell size and a periductal lymphoid infiltrate.[68]

Activation of c-erbB-2 has not been identified in benign breast lesions, including fibrocystic disease, fibroadenomas, and radial scars (Table 2). Strong membrane immunohistochemical reactivity for c-erbB-2 has not been described in atypical ductal hyperplasia, although weak accentuation of membrane staining has been noted infrequently.[39,42,54] In normal breast tissue, c-erbB-2 DNA is diploid, and c-erbB-2 is expressed at lower levels than in activated tumors.[34,35,65,88]

These preliminary data suggest that c-erbB-2 activation may not be useful for resolving many of the common problems in diagnostic surgical pathology. For example, c-erbB-2 activation is infrequent in tubular carcinoma and radial scars. In addition, because c-erbB-2 activation is unusual in atypical ductal hyperplasia, cribriform carcinoma in situ, and papillary carcinoma in situ, detection of c-erbB-2 activation in these lesions may not be helpful in their differential diagnosis. The histological features of comedocarcinoma in situ, which commonly overproduces c-erbB-2, are unlikely to be mistaken for those of benign lesions. Activation of

TABLE 2. c-erbB-2 ACTIVATION IN BENIGN HUMAN BREAST LESIONS

Histological Diagnosis	c-erbB-2 DNA Amplification[a]	c-erbB-2 mRNA Overproduction	c-erbB-2 Protein Overproduction
Fibrocystic disease	0/10[33]	—	0/32,[39] 0/9,[58] 0/8[65]
Atypical ductal hyperplasia	—	—	2(weak)/21,[54] 1(cytoplasmic)/13[39]
Benign ductal hyperplasia	—	—	0/12[39]
Sclerosing adenosis	—	—	0/4[39]
Fibroadenomas	0/16,[34] 0/6,[93] 0/2,[71] 0/1[91]	0/6,[35] 0/3[34]	0/21,[39] 0/10,[88] 0/6,[55] 0/3[42]
Radial scars	—	—	0/22[39]
Blunt duct adenosis	—	—	0/14[39]
"Breast mastosis"	—	0/3[35]	—

[a]Shown as number of cases with activation/number of cases studied; reference is given as a superscript.

c-erbB-2, however, does favor infiltrating ductal carcinoma over infiltrating lobular carcinoma. Further studies of these issues would be useful.

Correlation of c-erbB-2 Activation With Pathologic Prognostic Factors

Multiple studies have attempted to correlate c-erbB-2 activation with various pathologic prognostic factors (Table 3). Activation of c-erbB-2 was correlated with lymph node metastasis in 8 of 28 series, with higher histological grade in 6 of 17 series, and with higher stage in 4 of 14 series. Large tumor size was not associated with c-erbB-2 activation in most studies (11 of 14). Tetraploid DNA content and low proliferation, measured by Ki-67, have been suggested as prognostic factors and may correlate with c-erbB-2 activation.[6,7]

Correlation of c-erbB-2 Activation With Clinical-Prognostic Factors

Various studies have attempted also to correlate c-erbB-2 activation with clinical features that may predict a poor outcome (Table 4). Activation of c-erbB-2 correlated with absence of estrogen receptors in 10 of 28 series and with absence of progesterone receptors in 6 of 18 series. In most studies, patient age did not correlate with c-erbB-2 activation, and, in the rest of the reports, c-erbB-2 activation was associated with either younger or older ages.

Correlation of c-erbB-2 Activation With Patient Outcome

Slamon et al[79,81] first showed that amplification of the c-erbB-2 oncogene independently predicts decreased survival of patients with breast carcinoma. The correlation of c-erbB-2 amplification with poor outcome was nearly as strong as the correlation of number of involved lymph nodes with poor outcome. Slamon et al also reported that c-erbB-2 amplification is an important prognostic indicator only in patients with lymph node metastasis.[79,81]

A large number of subsequent studies also attempted to correlate c-erbB-2 activation with prognosis (Table 5). In 12 series, there was a correlation between c-erbB-2 activation and tumor recurrence or decreased survival. In five of these series, the predictive value of c-erbB-2 activation was reported to be independent of other prognostic factors. In contrast, 18 series did not confirm the correlation of c-erbB-2 activation with recurrence or survival. Four possible explanations for this controversy are discussed below.

One problem is that c-erbB-2 amplification correlates with prognosis mainly in patients with lymph node metastasis. As summarized in Table 5, most studies of patients with axillary lymph node metastasis showed a correlation of c-erbB-2 activation with poor outcome. In contrast, most studies of patients without axillary metastasis have not demonstrated a correlation with patient outcome. Table 6 summarizes the studies in which all patients (with and without axillary metastasis) were considered as one group. There is a trend for studies with a higher percentage of metastatic cases to show an association between c-erbB-2 activation and poor outcome. Thus, most of the current evidence suggests that c-erbB-2 activation has prognostic value only in patients with metastasis to lymph nodes.

TABLE 3. CORRELATION OF c-erbB-2 ACTIVATION WITH PATHOLOGIC PROGNOSTIC FACTORS IN BREAST CARCINOMA

Prognostic Factor	P[a]	c-erbB-2 DNA Amplification[b]	c-erbB-2 mRNA Overproduction	c-erbB-2 Protein Overproduction[c]
Metastasis to axillary lymph nodes	<0.05	(118)[95] (105)[94] (49)[21]	(104)[36] (92)[94] (9)[81]	(350)[159] (36)[13]
	0.05–0.15	(103)[79] (86)[79] (58)[111]	(50)[50]	(189)[92]
	>0.15	(279)[17] (176)[97] (157)[113]		(329)[170] (261)[96] (185)[111]
		(122)[4] (85)[90] (50)[52]		(185)[101] (102)[99] (50)[320]
		(50)[44] (47)[73] (41)[93]		
Larger size	<0.05	(280)[17]		(330)[170] (189)[92]
	0.05–0.15	(86)[79]		—
	>0.15	(178)[97] (157)[113] (103)[79]	(51)[50]	(350)[159] (185)[101] (34)[52]
		(64)[77] (58)[111] (45)[51]		
Higher stage	<0.05	(300)[17] (64)[77] (58)[111]		(349)[170]
		(56)[52]		
	0.05–0.15	(176)[97] (157)[113] (84)[90]		—
		(61)[50] (59)[13] (52)[67]		
		(41)[93]		
	>0.15		—	(102)[99] (56)[320]
Higher histological grade	<0.05	(47)[13] (15)[51]	(53)[95]	(176)[101] (168)[11] (38)[13]
	0.05–0.15	—		
	>0.15	(122)[4] (113)[94] (95)[90]	(86)[93] (65)[95]	(290)[96] (189)[52] (102)[99]
		(58)[111] (50)[44] (41)[93]		

[a] A correlation is statistically significant at <0.05, equivocal at best between 0.05 and 0.15, and not statistically significant at >0.15.
[b] Numbers inside parentheses are the number of patients in an individual study; superscript is the reference. Some studies analyzed more than one group of patients.
[c] By Western blot method; all other protein studies used immunohistochemical methods.

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TABLE 4. CORRELATION OF c-erbB-2 ACTIVATION WITH CLINICAL PROGNOSTIC FACTORS IN BREAST CARCINOMA

Prognostic Factor	P[a]	c-erbB-2 DNA Amplification[b]	c-erbB-2 mRNA Overproduction	c-erbB-2 Protein Overproduction[c]
Absence of estrogen receptors	<0.05	(253)[109] (141)[35] (109)[34] (86)[79] (50)[44] (47)[13]	(104)[35]	(350)[65c] (330)[172] (185)[101]
	0.05–0.15			
	>0.15	(157)[113] (122)[4] (103)[79] (95)[90] (64)[77] (61)[90] (58)[111] (53)[21] (51)[82] (41)[88]	(180)[89] (62)[65] (62)[95] (57)[90]	(290)[89] (172)[11] (51)[82] (38)[73]
Absence of progesterone receptors	<0.05	(253)[109] (141)[35] (109)[34] (50)[44]	—	(350)[65c] (305)[176]
	0.05–0.15	(86)[79] (49)[82]	—	(90)[11] (49)[82]
	>0.15	(157)[113] (122)[4] (103)[79] (64)[77]	(180)[89] (103)[95] (82)[93] (56)[35]	—
Age (menopausal status)	<0.05	(younger: 86)[79]	— [7?]	(younger: 330)[172] (older: 58)[32c]
	0.05–0.15	(230)[17] (176)[57] (157)[113] (122)[4] (116)[34] (103)[79] (95)[90] (64)[77] (58)[111] (56)[52] (53)[21] (49)[13] (41)[33] (15)[91]	(82)[65]	(350)[65c] (290)[89] (189)[32] (162)[11] (45)[82]
	>0.15			

[a] A correlation is statistically significant at <0.05, equivocal at best between 0.05 and 0.15, and not statistically significant at >0.15.
[b] Numbers inside parentheses are the number of patients in an individual study; superscript is the reference. Some studies analyzed more than one group of patients.
[c] By Western blot method; all other protein studies used immunohistochemical methods.

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TABLE 5. CORRELATION OF c-erbB-2 ACTIVATION WITH OUTCOME IN PATIENTS WITH BREAST CARCINOMA

P[a]	Type of c-erbB-2 Activation[b]	Number of Patients			Statistical Analysis[c]	Reference
		Total	With Metastasis to Axillary Lymph Nodes	No Metastasis		
<0.05	DNA	176			M	87
<0.05	DNA	61			U	50
<0.05	DNA	57			U	65
<0.05	DNA	41			U	93
<0.05	mRNA	62			U	65
<0.05	Protein	102			M	101
<0.05	DNA		345		M	81
<0.05	DNA		120		U	17
<0.05	DNA		91		U	87
<0.05	DNA		88		M	79
<0.05	Protein-WB		350		M	85
<0.05	Protein		62	44	U	101
0.05–0.15	DNA	57			U	111
0.05–0.15	Protein	189			M	92
0.05–0.15	Protein		120		U	86
>0.15	DNA	130			U	113
>0.15	DNA	122			M	4
>0.15	DNA	50			U	44
>0.15	mRNA	57			U	50
>0.15	Protein	290			M	86
>0.15	Protein	195			U	11
>0.15	Protein	102			U	39
>0.15	Protein		137		U	17
>0.15	DNA			181	M	81
>0.15	DNA			159	U	17
>0.15	DNA			73	U	87
>0.15	Protein-WB			378	U	85
>0.15	Protein-WB			192	U	17
>0.15	Protein			141	U	86
>0.15	Protein			41	U	40

[a]The endpoints of these studies were tumor recurrence or decreased survival or both. Correlation between c-erbB-2 activation and a poorer patient outcome is statistically significant at <0.05, is of equivocal significance at 0.05 to 0.15, and is not significant at >0.15.

[b]Shown as variable measured. Letters "WB" indicate assay by Western blot; the other protein studies used immunohistochemical methods.

[c]M = multivariate statistical analysis; U = univariate statistical analysis.

TABLE 6. PERCENTAGE OF BREAST CARCINOMAS WITH METASTASIS COMPARED WITH PROGNOSTIC SIGNIFICANCE OF c-erbB-2 ACTIVATION

% of tumors with lymph node metastasis in each study

71 (DNA)[90]

61 (DNA)[60]   64(DNA)[111]   64 (mRNA)[50]
59 (DNA)[67]   61(DNA)[4]
58 (Protein)[101]

                                 58 (DNA)[44]
                                 57 (DNA)[113]
                                 55(Protein)[39]

                                 48(Protein)[11]
                                 46(Protein)[86]

                 42(Protein)[92]

P<0.05   0.05<P<0.15   P>0.15
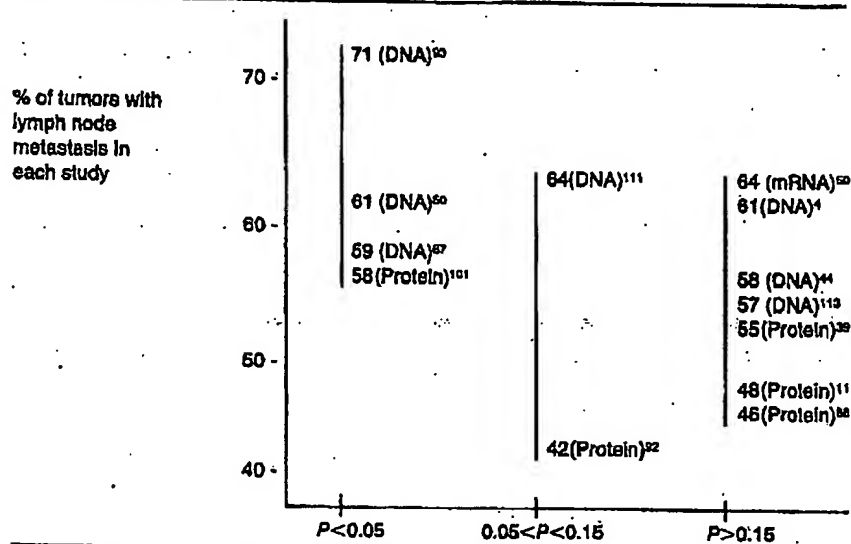
P for correlation of c-erbB-2 activation with patient outcome.
Each study's percentage of breast carcinomas with metastasis is compared with the correlation between c-erbB-2 activation and outcome. These data include only those studies that considered, as one group, all breast cancer patients, whether or not they had axillary metastasis. Superscripts are the references. In parentheses are the types of c-erbB-2 activation. P values are interpreted as in Table 3.

A second problem is that various types of breast carcinoma are grouped together in many survival studies. Because the current literature suggests that c-erbB-2 activation is infrequent in lobular carcinoma, studies that combine infiltrating ductal and lobular carcinomas may dilute the prognostic effect of c-erbB-2 activation in ductal tumors. In addition, most studies do not analyze inflammatory breast carcinoma separately. This condition frequently shows c-erbB-2 activation and has a worse prognosis than the usual mammary carcinoma, but it is an uncommon lesion.

A third potential problem is the paucity of studies that attempt to correlate c-erbB-2 activation with clinical outcome in subsets of breast carcinoma without metastasis. Two recent abstracts reported that in patients without lymph node metastasis who had various risk factors for recurrence (such as large tumor size and absence of estrogen receptors), c-erbB-2 overexpression predicted early recurrence.[23,67] In patients with ductal carcinoma in situ, one small study found no association between tumor recurrence and c-erbB-2 activation.[40]

A fourth problem is the lack of data regarding whether the prognosis correlates better with c-erbB-2 DNA amplification or with mRNA or protein overproduction. Most studies that find a correlation between c-erbB-2 activa-

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tion and poor patient outcome measure c-*erb*B-2 DNA amplification (Table 5), and breast carcinoma patients with greater amplification of c-*erb*B-2 may have poorer survival.[79,81] Recent studies suggest that amplification has more prognostic power than overproduction,[17,34,35] but the clinical significance of c-*erb*B-2 overproduction without DNA amplification deserves further research.[17,52] Few studies have attempted to correlate patient outcome with c-*erb*B-2 mRNA overproduction, and many studies of c-*erb*B-2 protein overproduction use relatively less reliable methods such as immunohistochemical studies with polyclonal antibodies.

Comparison of c-*erb*B-2 Activation With Other Oncogenes in Breast Carcinoma

Other oncogenes that may have prognostic implications in human breast cancer are reviewed elsewhere.[71,106] This section will be restricted to a comparison between the clinical relevance of c-*erb*B-2 and these other oncogenes.

The c-*myc* gene is often activated in breast carcinomas, but c-*myc* activation generally has less prognostic importance than c-*erb*B-2 activation.[21,34,77,87,90] One study found a correlation between increased mRNAs of c-*erb*B-2 and c-*myc*, although other reports have not confirmed this.[34,106] Subsequent research, however, could demonstrate a subset of breast carcinomas in which c-*myc* has more prognostic importance than c-*erb*B-2.

The gene c-*erb*B-1 for the epidermal growth factor receptor (EGFR) is homologous with c-*erb*B-2 but is infrequently amplified in breast carcinomas.[79] Overproduction of EGFR, however, occurs more frequently than amplification and may correlate with a poor prognosis. In studies that have examined both c-*erb*B-2 and EGFR in the same tumor, c-*erb*B-2 has a stronger correlation with poor prognostic factors.[35,52] Studies have tended to show no correlation between amplification of c-*erb*B-2 and c-*erb*B-1 or overproduction of c-*erb*B-2 and EGFR, although at the molecular level EGFR mediates phosphorylation of c-*erb*B-2 protein.[51,52,61,88,100] Recent reviews describe EGFR in breast carcinoma.[49,100]

The genes c-*erb*A and *ear*-1 are homologous to the thyroid hormone receptor, and they are located adjacent to c-*erb*B-2 on chromosome 17. These genes are frequently coamplified with c-*erb*B-2 in breast carcinomas. The absence of c-*erb*A expression in breast carcinomas, however, is evidence against an important role for this gene in breast neoplasia.[90] Amplification of c-*erb*B-2 can occur without *ear*-1 amplification, and these tumors have a decreased survival that is similar to tumors with both c-*erb*B-2 and *ear*-1 amplification.[67] Consequently, c-*erb*B-2 amplification seems to be more important than amplification of c-*erb*A or *ear*-1.

Other genes also have been compared with c-*erb*B-2 activation in breast carcinomas. One study found a significant correlation between increased c-*erb*B-2 mRNA and increased mRNAs of *fos*, platelet-derived growth factor chain A, and Ki-*ras*.[106] Allelic deletion of c-Ha-*ras* may indicate a poorer prognosis in breast carcinoma,[21] but it has not been compared with c-*erb*B-2 activation. Some studies have suggested a correlation between advanced stage or recurrence of breast carcinoma and activation of any one of several oncogenes.[21,113]

ACTIVATION OF c-erbB-2 IN NON-MAMMARY TISSUES

Incidence of c-erbB-2 Activation in Non-Mammary Tissues

Table 7 summarizes the normal tissues in which c-erbB-2 expression has been detected, usually with immunohistochemical methods using polyclonal anti-

TABLE 7. PRESENCE OR ABSENCE OF c-erbB-2 mRNA OR c-erbB-2 PROTEIN IN NORMAL HUMAN TISSUES

Tissues With c-erbB-2 mRNA	Tissues Producing c-erbB-2 Protein[a]	Tissues Lacking c-erbB-2 mRNA	Tissues Lacking c-erbB-2 Protein
Skin[24]	Epidermis[56]		
	External root sheath[56]		
	Eccrine sweat gland[56]		
	Fetal oral mucosa[62]		Postnatal oral mucosa[62]
	Fetal esophagus[62]		Postnatal esophagus[62]
Stomach[24]	Stomach[22,62]		
	Fetal intestine[62a]		
Jejunum[24]	Small intestine[22,62]		
Colon[24]	Colon[22,62]		
Kidney[24]	Fetal kidney[62a]	Kidneys[104]	Glomerulus[62]
			Postnatal Bowman's capsule[62]
	Fetal proximal tubule[62]		Postnatal proximal tubule[62]
	Distal tubule[62]		
	Fetal collecting duct[62]		Postnatal collecting duct[62]
	Fetal renal pelvis[62]		Postnatal renal pelvis[62]
	Fetal ureter[62]		Postnatal fetal ureter[62]
Liver[24]	Hepatocytes[22]		Liver[62,65]
	Pancreatic acini[22]		
	Pancreatic ducts[22,62]		
	Endocrine cells of islets of Langerhans[22]		Pancreatic islets[62]
Lung[24]	Fetal trachea[62]		Postnatal trachea[62]
	Fetal bronchioles[62]		Postnatal bronchioles[62]
	Bronchioles[99]		
			Postnatal alveoli[62,99]
Fetal brain[24]			Postnatal brain[62]
	Fetal ganglion cells[62]		Postnatal ganglion cells[62]
Thyroid[1]			
Uterus[24]			
	Ovary[12]		
	Blood vessels[62]		Endothelium[62]
Placenta[24]			
			Adrenocortical cells[62]
			Postnatal thymus[62]
			Fibroblasts[62]
			Smooth muscle cells[62]
			Cardiac muscle cells[62]

[a]This protein study used Western blots; the rest used immunohistochemical methods.

bodies. Only a few studies have been performed, and some of these do not demonstrate convincing cell membrane reactivity in the published photographs. The interpretations in these studies, however, are listed, with the caveat that these findings should be confirmed by immunoprecipitation or Western or RNA blots. Production of c-erbB-2 has been identified in normal epithelium of the gastrointestinal tract and skin. Discrepancies regarding c-erbB-2 protein in other tissues could be due, at least in part, to differences in techniques.

The data on c-erbB-2 activation in various non-mammary neoplasms should be interpreted with caution, because only small numbers of tumors have been studied, usually by immunohistochemical methods using polyclonal antibodies. Studies using cell lines have been excluded, because cell culture can induce amplification and overexpression of other genes, although this has not been documented for c-erbB-2.

Activation of c-erbB-2 has been identified in 32 percent (64 of 203) of ovarian carcinomas in eight studies (Table 8). One abstract[45] stated that ovarian carcinomas contained significantly more c-erbB-2 protein than ovarian non-epithelial malignancies. Another report[81] showed that 12 percent of ovarian carcinomas had c-erbB-2 overproduction without amplification.

Activation of c-erbB-2 has been identified in 20 percent (40 of 198) of gastric adenocarcinomas in seven studies, including 33 percent (21 of 64) of

TABLE 8. c-erbB-2 ACTIVATION IN HUMAN GYNECOLOGIC TUMORS[a]

Tumor Type	c-erbB-2 DNA Amplification	c-erbB-2 mRNA Overproduction	c-erbB-2 Protein Overproduction
Ovary—carcinoma, not otherwise specified	31/120,[81] 1/11,[57] 0/5,[107] 0/5,[64] 0/3,[112] 0/2,[72] 0/1[110]	23/67[81]	23/73,[12] 36/72[81]
Ovary—serous (papillary) carcinoma	2/7,[110] 1/7,[112] 0/6[72]	—	—
Ovary—endometrioid carcinoma	0/3[110]	—	—
Ovary—mucinous carcinoma	1/2,[110] 0/1[72]	—	—
Ovary—clear cell carcinoma	0/2,[112] 0/1[72]	—	—
Ovary—mixed epithelial carcinoma	0/2[72]	—	—
Ovary—endometrioid borderline tumor	0/1[72]	—	—
Ovary—mucinous borderline tumor	0/3[72]	—	—
Ovary—serous cystadenoma	0/4[72]	—	—
Ovary—mucinous cystadenoma	0/2[72]	—	—
Ovary—sclerosing stromal tumor	0/1[72]	—	—
Ovary—fibrothecoma	0/1[72]	—	—
Uterus—endometrial adenocarcinoma	0/4,[64] 0/1[110]	—	—

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods.

intestinal or tubular subtypes and 9 percent (4 of 47) of diffuse or signet ring cell subtypes (Table 9). Activation of c-erbB-2 has been detected in 2 percent (6 of 281) of colorectal carcinomas, although an additional immunohistochemical study detected c-erbB-2 protein in seven of eight tissues fixed in Bouin's solution. One study found greater immunohistochemical reactivity for c-erbB-2 protein in colonic adenomatous polyps than in the adjacent normal epithelium, using Bouin's fixative. Lesions with anaplastic features and progression to invasive carcinoma tended to show decreased immunohistochemical reactivity for c-erbB-2 protein.[23] Hepatocellular carcinomas (12 of 14 cases) and cholangiocarcinomas (46 of 63 cases) reacted with antibodies against c-erbB-2 in one study, but some of these "positive" cases showed only diffuse cytoplasmic staining, which

TABLE 9. c-erbB-2 ACTIVATION IN HUMAN GASTROINTESTINAL TUMORS[a]

Tumor Type	c-erbB-2 DNA Amplification	c-erbB-2 Protein Over-production
Esophagus—squamous cell carcinoma	0/1[107]	0/1[61]
Stomach—carcinoma, poorly differentiated	0/22[108]	—
Stomach—adenocarcinoma	2/24,[94] 2/9,[107] 2/8,[111] 2/8,[57] 0/1[108]	4/27,[29] 3/10[61]
Stomach—carcinoma, intestinal or tubular type	5/10[108]	16/54[29]
Stomach—carcinoma, diffuse or signet ring cell type	0/2[108]	4/45[29]
Colorectum—carcinoma	2/49,[94] 1/45,[111] 1/45,[57] 1/45,[60] 0/40,[61] 0/32,[107] 0/3[52]	1/22,[58] 7/8[22b]
Colon—villous adenoma	0/1[60]	—
Colon—tubulovillous adenoma	0/5[60]	—
Colon—tubular adenoma	0/7[60]	19/19[22b]
Colon—hyperplastic polyp	0/1[60]	—
Intestine—leiomyosarcoma	—	0/1[61]
Hepatocellular carcinoma	0/12[111]	12/14,[36] 0/2[61]
Hepatoblastoma	0/1[57]	—
Cholangiocarcinoma	—	46/63[65]
Pancreas—adenocarcinoma	—	2/80,[41c] 0/2[61]
Pancreas—acinar carcinoma	—	0/1[41]
Pancreas—clear cell carcinoma	—	0/2[41]
Pancreas—large cell carcinoma	—	0/3[41]
Pancreas—signet ring carcinoma	—	0/1[41]
Pancreas—chronic inflammation	—	0/14[41c]

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods. No studies analyzed for c-erbB-2 mRNA.
[b]Tissues fixed in Bouin's solution.
[c]Only cases with distinct membrane staining are interpreted as showing c-erbB-2 overproduction.

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TABLE 10. c-erbB-2 ACTIVATION IN HUMAN PULMONARY TUMORS[a]

Tumor Type	c-erbB-2 DNA Amplification	c-erbB-2 Protein Overproduction
Non-small cell carcinoma	2/60,[75] 0/60[61]	1/84[59]
Epidermoid carcinoma	0/13,[62] 0/10,[67] 0/6[20]	3/5[99]
Adenocarcinoma	0/21,[62] 1/13,[20] 0/7,[111] 0/7,[67] 0/3[107]	4/12[99]
Large cell carcinoma	0/9,[62] 0/6[20]	—
Small cell carcinoma	—	0/26,[59] 0/3[99]
Carcinoid tumor	0/1[62]	0/3[99]

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods. No studies analyzed for c-erbB-2 mRNA.

does not indicate c-erbB-2 activation in breast neoplasms.[95] Also, some pancreatic carcinomas and chronic pancreatitis tissue had cytoplasmic immunohistochemical reactivity for c-erbB-2 protein, in addition to the rare case of pancreatic adenocarcinoma with distinct cell membrane staining.[41]

Tables 10 through 14 summarize the studies of c-erbB-2 activation in other neoplasms. The c-erbB-2 oncogene is not activated in most of these tumors. Activation of c-erbB-2 has been detected in 1 percent (4 of 299) of pulmonary non-small cell carcinomas in nine studies, although one additional report[99] found c-erbB-2 protein overproduction in 41 percent (7 of 17). Renal cell carcinoma had c-erbB-2 activation in 7 percent (2 of 30) in four studies. Overproduction of c-erbB-2 protein was described in one transitional cell carcinoma of the urinary bladder, a grade 2 papillary lesion.[68] Squamous cell carcinoma and basal cell carcinoma of the skin may contain c-erbB-2 protein, but it is not clear

TABLE 11. c-erbB-2 ACTIVATION IN HUMAN HEMATOLOGIC PROLIFERATIONS[a]

Tumor Type	c-erbB-2 DNA Amplification	c-erbB-2 mRNA Overproduction	c-erbB-2 Protein Overproduction
Hematologic malignancies	0/23[111]	—	—
Malignant lymphoma	0/9,[57] 0/3[107]	0/1[1]	0/15[61]
Acute leukemia	0/14[57]	—	—
Acute lymphoblastic leukemia	0/1[107]	—	—
Acute myeloblastic leukemia	0/3[107]	—	—
Chronic leukemia	0/19[57]	—	—
Chronic lymphocytic leukemia	0/6[197]	—	—
Chronic myelogenous leukemia	0/8[107]	—	—
Myeloproliferative disorder	0/1[57]	—	—

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods.

TABLE 12. c-erbB-2 ACTIVATION IN HUMAN TUMORS OF SOFT TISSUE AND BONE[a]

Tumor Type	c-erbB-2 DNA Amplification
Sarcoma	0/10,[111] 0/8[57]
Malignant fibrous histiocytoma	0/1[107]
Liposarcoma	0/3[107]
Pleomorphic sarcoma	0/1[107]
Rhabdomyosarcoma	0/1[107]
Osteogenic sarcoma	0/2,[107] 0/2[57]
Chondrosarcoma	0/1[107]
Ewing's sarcoma	0/1[57]
Schwannoma	0/1[57]

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. No studies analyzed for c-erbB-2 mRNA or c-erbB-2 protein.

whether the protein level is increased over that of normal skin.[56] Thyroid carcinomas and adenomas can have low levels of increased c-erbB-2 mRNA. One abstract described low-level c-erbB-2 DNA amplification in one of ten salivary gland pleomorphic adenomas.[48]

Correlation of c-erbB-2 Activation With Patient Outcome

Very few studies have attempted to correlate c-erbB-2 activation in non-mammary tumors with outcome. Slamon et al[91] showed that c-erbB-2 amplification or overexpression in ovarian carcinomas correlates with decreased survival, especially when marked activation is present. However, they did not report the stage, histological grade, or histological subtype of these neoplasms. Another study of stages III and IV ovarian carcinomas found a correlation between decreased survival and c-erbB-2 protein overproduction, but not between survival and histological grade.[12] One abstract stated that c-erbB-2 protein overproduction in 10 of 16 pulmonary adenocarcinomas correlated with decreased disease-free interval.[70] Another abstract described a tendency for immunohisto-

TABLE 13. c-erbB-2 ACTIVATION IN HUMAN TUMORS OF THE URINARY TRACT[a]

Tumor Type	c-erbB-2 DNA Amplification	c-erbB-2 mRNA Over-production	c-erbB-2 Protein Over-production
Kidney—renal cell carcinoma	1/5,[57] 1/4,[107] 0/5[94]	0/16[104]	—
Wilms' tumor	0/4[57]	—	—
Prostate—adenocarcinoma	—	—	0/23[59]
Urinary bladder—carcinoma	—	—	1/48[59]

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods.

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TABLE 14. c-erbB-2 ACTIVATION IN MISCELLANEOUS HUMAN TUMORS[a]

Tumor Type	c-erbB-2 DNA Amplification	c-erbB-2 mRNA Overproduction	c-erbB-2 Protein Over-production
Skin—malignant melanoma	—	—	0/10[98]
Skin, head and neck—squamous cell carcinoma	0/7[107]	—	—
Site not stated—squamous cell carcinoma	0/8,[57] 0/2[76]	—	—
Salivary gland—adenocarcinoma	1/1[76]	—	—
Parotid gland—adenoid cystic carcinoma	—	—	0/1[51]
Thyroid—anaplastic carcinoma	0/1[1]	0/1[1]	—
Thyroid—papillary carcinoma	0/5[1]	3(low levels)/5[1]	—
Thyroid—adenocarcinoma	0/1[34]	—	—
Thyroid—adenoma	0/2[1]	1(low levels)/2[1]	—
Neuroblastoma	0/35,[61] 0/9,[57] 0/1[76]	—	—
Meningioma	0/2[57]	—	—

[a]Shown as number of cases with amplification (or overproduction)/total number of cases studied; reference is given as superscript. All protein studies used immunohistochemical methods.

chemical reactivity for c-erbB-2 protein to correlate with higher grades of prostatic adenocarcinoma.[97] Additional prognostic studies of ovarian carcinomas and other neoplasms are needed.

SUMMARY

Activation of the c-erbB-2 oncogene can occur by amplification of c-erbB-2 DNA and by overproduction of c-erbB-2 mRNA and c-erbB-2 protein. Approximately 20 percent of breast carcinomas show evidence of c-erbB-2 activation, which correlates with a poor prognosis primarily in patients with metastasis to axillary lymph nodes. Studies that have attempted to correlate c-erbB-2 activation with other prognostic factors in breast carcinoma have reported conflicting conclusions. The pathologic and clinical significance of c-erbB-2 activation in other neoplasms is unclear and should be assessed by additional studies.

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Exhibit 2

GNE.3230R1C39 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Eaton, et al.
Appl. No.	:	10/063,557
Filed	:	May 2, 2002
For	:	SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING THE SAME
Examiner	:	David J. Blanchard
Group Art Unit	:	1642

DECLARATION OF J. CHRISTOPHER GRIMALDI, UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, J. Christopher Grimaldi, declare and say as follows:

1.   I am a Senior Research Associate in the Molecular Biology Department of Genentech, Inc., South San Francisco, CA 94080.

2.   I joined Genentech in January of 1999. From 1999 to 2003, I directed the Cloning Laboratory in the Molecular Biology Department. During this time I directed or performed numerous molecular biology techniques including qualitative Polymerase Chain Reaction (PCR) analyses. I am currently involved in, among other projects, the isolation of genes coding for membrane associated proteins which can be used as targets for antibody therapeutics against cancer. In connection with the above-identified patent application, I personally performed or directed the semi-quantitative PCR analyses in the assay entitled "Tumor Versus Normal Differential Tissue Expression Distribution" which is described in EXAMPLE 18 in the specification that were used to identify differences in gene expression between tumor tissue and their normal counterparts.

3.   My scientific Curriculum Vitae, including my list of publications, is attached to and forms part of this Declaration (Exhibit A).

4.   In differential gene expression studies, one looks for genes whose expression levels differ significantly under different conditions, for example, in normal versus diseased tissue.

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Chromosomal aberrations, such as gene amplification, and chromosomal translocations are important markers of specific types of cancer and lead to the aberrant expression of specific genes and their encoded polypeptides, including over-expression and under-expression. For example, gene amplification is a process in which specific regions of a chromosome are duplicated, thus creating multiple copies of certain genes that normally exist as a single copy. Gene under-expression can occur when a gene is not transcribed into mRNA. In addition, chromosomal translocations occur when two different chromosomes break and are rejoined to each other chromosome resulting in a chimeric chromosome which displays a different expression pattern relative to the parent chromosomes. Amplification of certain genes such as Her2/Neu [Singleton *et al.*, Pathol. Annu., 27Pt1:165-190], or chromosomal translocations such as t(5;14), [Grimaldi *et al.*, Blood, 73(8):2081-2085(1989); Meeker *et al.*, Blood, 76(2):285-289(1990)] give cancer cells a growth or survival advantage relative to normal cells, and might also provide a mechanism of tumor cell resistance to chemotherapy or radiotherapy. When the chromosomal aberration results in the aberrant expression of a mRNA and the corresponding gene product (the polypeptide), as it does in the aforementioned cases, the gene product is a promising target for cancer therapy, for example, by the therapeutic antibody approach.

5.   Comparison of gene expression levels in normal versus diseased tissue has important implications both diagnostically and therapeutically. For example, those who work in this field are well aware that in the vast majority of cases, when a gene is over-expressed, as evidenced by an increased production of mRNA, the gene product or polypeptide will also be over-expressed. It is unlikely that one identifies increased mRNA expression without associated increased protein expression. This same principle applies to gene under-expression. When a gene is under-expressed, the gene product is also likely to be under-expressed. Stated in another way, two cell samples which have differing mRNA concentrations for a specific gene are expected to have correspondingly different concentration of protein for that gene. Techniques used to detect mRNA, such as Northern Blotting, Differential Display, *in situ* hybridization, quantitative PCR, Taqman, and more recently Microarray technology all rely on the dogma that a change in mRNA will represent a similar change in protein. If this dogma did not hold true then these techniques would have little value and not be so widely used. The use of mRNA quantitation techniques have identified a seemingly endless number of genes which are differentially expressed in various tissues and these genes have subsequently been shown to have correspondingly similar changes in their protein levels. Thus, the detection of increased mRNA expression is expected to result in increased polypeptide expression, and the detection of decreased mRNA expression is expected to result in decreased polypeptide expression. The detection of increased or decreased polypeptide expression can be used for cancer diagnosis and treatment.

6.   However, even in the rare case where the protein expression does not correlate with the mRNA expression, this still provides significant information useful for cancer diagnosis and treatment. For example, if over- or under-expression of a gene product does not correlate with over- or under-expression of mRNA in certain tumor types but does so in others, then identification of both gene expression and protein expression enables more accurate tumor classification and hence better determination of suitable therapy. In addition, absence of over- or

under-expression of the gene product in the presence of a particular over- or under-expression of mRNA is crucial information for the practicing clinician. For example, if a gene is over-expressed but the corresponding gene product is not significantly over-expressed, the clinician accordingly will decide not to treat a patient with agents that target that gene product.

7.   I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

By: _____   Date: _____
    J. Christopher Grimaldi

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DECLARATION OF PAUL POLAKIS, Ph.D.

I, Paul Polakis, Ph.D., declare and say as follows:

1. I was awarded a Ph.D. by the Department of Biochemistry of the Michigan State University in 1984. My scientific Curriculum Vitae is attached to and forms part of this Declaration (Exhibit A).

2. I am currently employed by Genentech, Inc. where my job title is Staff Scientist. Since joining Genentech in 1999, one of my primary responsibilities has been leading Genentech's Tumor Antigen Project, which is a large research project with a primary focus on identifying tumor cell markers that find use as targets for both the diagnosis and treatment of cancer in humans.

3. As part of the Tumor Antigen Project, my laboratory has been analyzing differential expression of various genes in tumor cells relative to normal cells. The purpose of this research is to identify proteins that are abundantly expressed on certain tumor cells and that are either (i) not expressed, or (ii) expressed at lower levels, on corresponding normal cells. We call such differentially expressed proteins "tumor antigen proteins". When such a tumor antigen protein is identified, one can produce an antibody that recognizes and binds to that protein. Such an antibody finds use in the diagnosis of human cancer and may ultimately serve as an effective therapeutic in the treatment of human cancer.

4. In the course of the research conducted by Genentech's Tumor Antigen Project, we have employed a variety of scientific techniques for detecting and studying differential gene expression in human tumor cells relative to normal cells, at genomic DNA, mRNA and protein levels. An important example of one such technique is the well known and widely used technique of microarray analysis which has proven to be extremely useful for the identification of mRNA molecules that are differentially expressed in one tissue or cell type relative to another. In the course of our research using microarray analysis, we have identified approximately 200 gene transcripts that are present in human tumor cells at significantly higher levels than in corresponding normal human cells. To date, we have generated antibodies that bind to about 30 of the tumor antigen proteins expressed from these differentially expressed gene transcripts and have used these antibodies to quantitatively determine the level of production of these tumor antigen proteins in both human cancer cells and corresponding normal cells. We have then compared the levels of mRNA and protein in both the tumor and normal cells analyzed.

5. From the mRNA and protein expression analyses described in paragraph 4 above, we have observed that there is a strong correlation between changes in the level of mRNA present in any particular cell type and the level of protein

expressed from that mRNA in that cell type. In approximately 80% of our observations we have found that increases in the level of a particular mRNA correlates with changes in the level of protein expressed from that mRNA when human tumor cells are compared with their corresponding normal cells.

6. Based upon my own experience accumulated in more than 20 years of research, including the data discussed in paragraphs 4 and 5 above and my knowledge of the relevant scientific literature, it is my considered scientific opinion that for human genes, an increased level of mRNA in a tumor cell relative to a normal cell typically correlates to a similar increase in abundance of the encoded protein in the tumor cell relative to the normal cell. In fact, it remains a central dogma in molecular biology that increased mRNA levels are predictive of corresponding increased levels of the encoded protein. While there have been published reports of genes for which such a correlation does not exist, it is my opinion that such reports are exceptions to the commonly understood general rule that increased mRNA levels are predictive of corresponding increased levels of the encoded protein.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

Dated: _5/07/0Y_   By: _Paul Polakis_

Paul Polakis, Ph.D.

CURRICULUM VITAE

PAUL G. POLAKIS
Staff Scientist
Genentech, Inc
1 DNA Way, MS#40
S. San Francisco, CA 94080

EDUCATION:

Ph.D., Biochemistry, Department of Biochemistry,
Michigan State University (1984)

B.S., Biology. College of Natural Science, Michigan State University (1977)

PROFESSIONAL EXPERIENCE:

2002-present	Staff Scientist, Genentech, Inc S. San Francisco, CA
1999- 2002	Senior Scientist, Genentech, Inc., S. San Francisco, CA
1997 -1999	Research Director Onyx Pharmaceuticals, Richmond, CA
1992- 1996	Senior Scientist, Project Leader, Onyx Pharmaceuticals, Richmond, CA
1991-1992	Senior Scientist, Chiron Corporation, Emeryville, CA.
1989-1991	Scientist, Cetus Corporation, Emeryville CA.
1987-1989	Postdoctoral Research Associate, Genentech, Inc., South SanFrancisco, CA.
1985-1987	Postdoctoral Research Associate, Department of Medicine, Duke University Medical Center, Durham, NC

1984-1985	Assistant Professor, Department of Chemistry, Oberlin College, Oberlin, Ohio
1980-1984	Graduate Research Assistant, Department of Biochemistry, Michigan State University East Lansing, Michigan

PUBLICATIONS:

1. **Polakis, P G.** and Wilson, J. E. 1982 Purification of a Highly Bindable Rat Brain Hexokinase by High Performance Liquid Chromatography. **Biochem. Biophys. Res. Commun.** 107, 937-943.

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13. Munemitsu, S., Innis, M.A., Clark, R., McCormick, F., Ullrich, A. and **Polakis, P.G.** 1990 Molecular Cloning and Expression of a G25K cDNA, the Human Homolog of the Yeast Cell Cycle Gene CDC42. **Mol. Cell. Biol**. 10, 5977-5982.

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22. **Polakis P**. and McCormick, F. 1992 Interactions Between p21^{ras} Proteins and Their GTPase Activating Proteins. In **Cancer Surveys** (Franks, L. M., ed.) 12, 25-42.

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MOLECULAR BIOLOGY OF
THE CELL
fourth edition

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gene A   gene B

]DNA

TRANSCRIPTION   TRANSCRIPTION

RNA   RNA

TRANSLATION   TRANSLATION

Figure 6–3 Genes can be expressed with different efficiencies. Gene A is transcribed and translated much more efficiently than gene B. This allows the amount of protein A in the cell to be much greater than that of protein B.

FROM DNA TO RNA

Transcription and translation are the means by which cells read out, or express, the genetic instructions in their genes. Because many identical RNA copies can be made from the same gene, and each RNA molecule can direct the synthesis of many identical protein molecules, cells can synthesize a large amount of protein rapidly when necessary. But each gene can also be transcribed and translated with a different efficiency, allowing the cell to make vast quantities of some proteins and tiny quantities of others (Figure 6–3). Moreover, as we see in the next chapter, a cell can change (or regulate) the expression of each of its genes according to the needs of the moment—most obviously by controlling the production of its RNA.

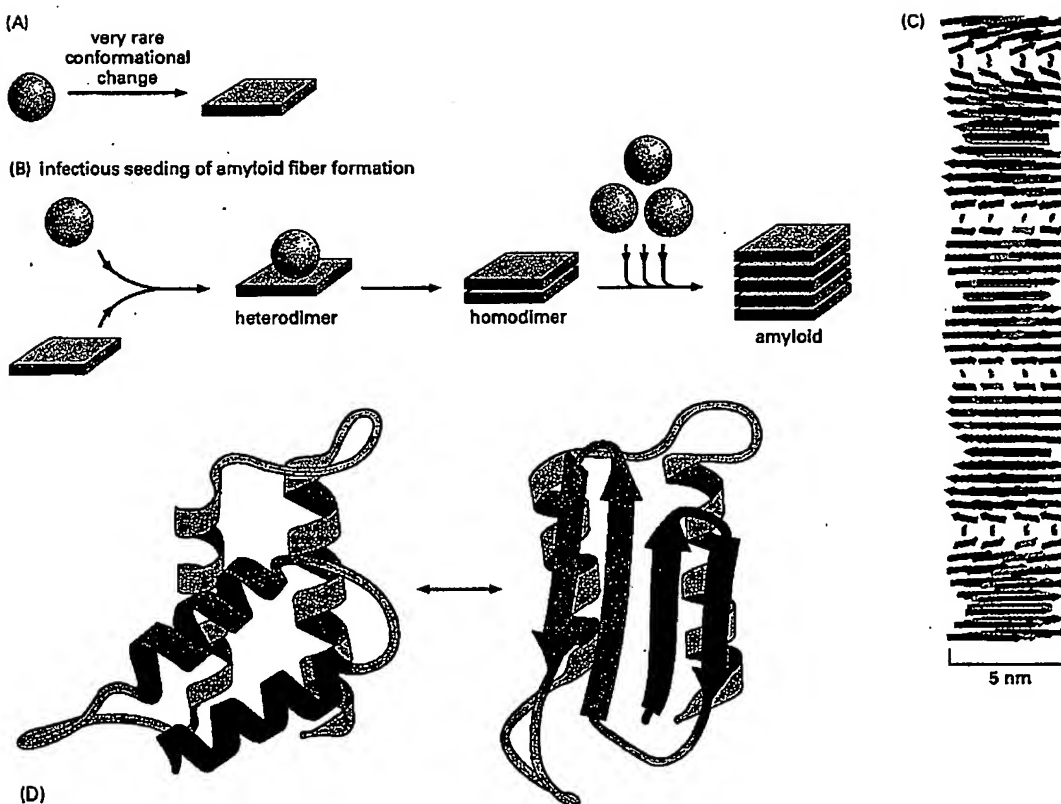## Portions of DNA Sequence Are Transcribed into RNA

The first step a cell takes in reading out a needed part of its genetic instructions is to copy a particular portion of its DNA nucleotide sequence—a gene—into an RNA nucleotide sequence. The information in RNA, although copied into another chemical form, is still written in essentially the same language as it is in DNA—the language of a nucleotide sequence. Hence the name **transcription**.

Like DNA, RNA is a linear polymer made of four different types of nucleotide subunits linked together by phosphodiester bonds (Figure 6–4). It differs from DNA chemically in two respects: (1) the nucleotides in RNA are *ribonucleotides*—that is, they contain the sugar ribose (hence the name *ribonucleic acid*) rather than deoxyribose; (2) although, like DNA, RNA contains the bases adenine (A), guanine (G), and cytosine (C), it contains the base uracil (U) instead of the thymine (T) in DNA. Since U, like T, can base-pair by hydrogen-bonding with A (Figure 6–5), the complementary base-pairing properties described for DNA in Chapters 4 and 5 apply also to RNA (in RNA, G pairs with C, and A pairs with U). It is not uncommon, however, to find other types of base pairs in RNA: for example, G pairing with U occasionally.

Despite these small chemical differences, DNA and RNA differ quite dramatically in overall structure. Whereas DNA always occurs in cells as a double-stranded helix, RNA is single-stranded. RNA chains therefore fold up into a variety of shapes, just as a polypeptide chain folds up to form the final shape of a protein (Figure 6–6). As we see later in this chapter, the ability to fold into complex three-dimensional shapes allows some RNA molecules to have structural and catalytic functions.

Transcription Produces RNA Complementary to One Strand of DNA

All of the RNA in a cell is made by DNA transcription, a process that has certain similarities to the process of DNA replication discussed in Chapter 5.

(A)

very rare
conformational
change

(B) infectious seeding of amyloid fiber formation

heterodimer   homodimer

amyloid

(C)

5 nm

(D)

Figure 6–89 Protein aggregates that cause human disease. (A) Schematic illustration of the type of conformational change in a protein that produces material for a cross-beta filament. (B) Diagram illustrating the self-infectious nature of the protein aggregation that is central to prion diseases. PrP is highly unusual because the misfolded version of the protein, called PrP*, induces the normal PrP protein it contacts to change its conformation, as shown. Most of the human diseases caused by protein aggregation are caused by the overproduction of a variant protein that is especially prone to aggregation, but because this structure is not infectious in this way, it cannot spread from one animal to another. (C) Drawing of a cross-beta filament, a common type of protease-resistant protein aggregate found in a variety of human neurological diseases. Because the hydrogen-bond interactions in a β sheet form between polypeptide backbone atoms (see Figure 3–9), a number of different abnormally folded proteins can produce this structure. (D) One of several possible models for the conversion of PrP to PrP*, showing the likely change of two α-helices into four β-strands. Although the structure of the normal protein has been determined accurately, the structure of the infectious form is not yet known with certainty because the aggregation has prevented the use of standard structural techniques. (C, courtesy of Louise Serpell, adapted from M. Sunde et al., J. Mol. Biol. 273:729–739, 1997; D, adapted from S.B. Prusiner, Trends Biochem. Sci. 21:482–487, 1996.)

animals and humans. It can be dangerous to eat the tissues of animals that contain PrP*, as witnessed most recently by the spread of BSE (commonly referred to as the "mad cow disease") from cattle to humans in Great Britain.

Fortunately, in the absence of PrP*, PrP is extraordinarily difficult to convert to its abnormal form. Although very few proteins have the potential to misfold into an infectious conformation, a similar transformation has been discovered to be the cause of an otherwise mysterious "protein-only inheritance" observed in yeast cells.

There Are Many Steps From DNA to Protein

We have seen so far in this chapter that many different types of chemical reactions are required to produce a properly folded protein from the information contained in a gene (Figure 6–90). The final level of a properly folded protein in a cell therefore depends upon the efficiency with which each of the many steps is performed.

We discuss in Chapter 7 that cells have the ability to change the levels of their proteins according to their needs. In principle, any or all of the steps in Fig-
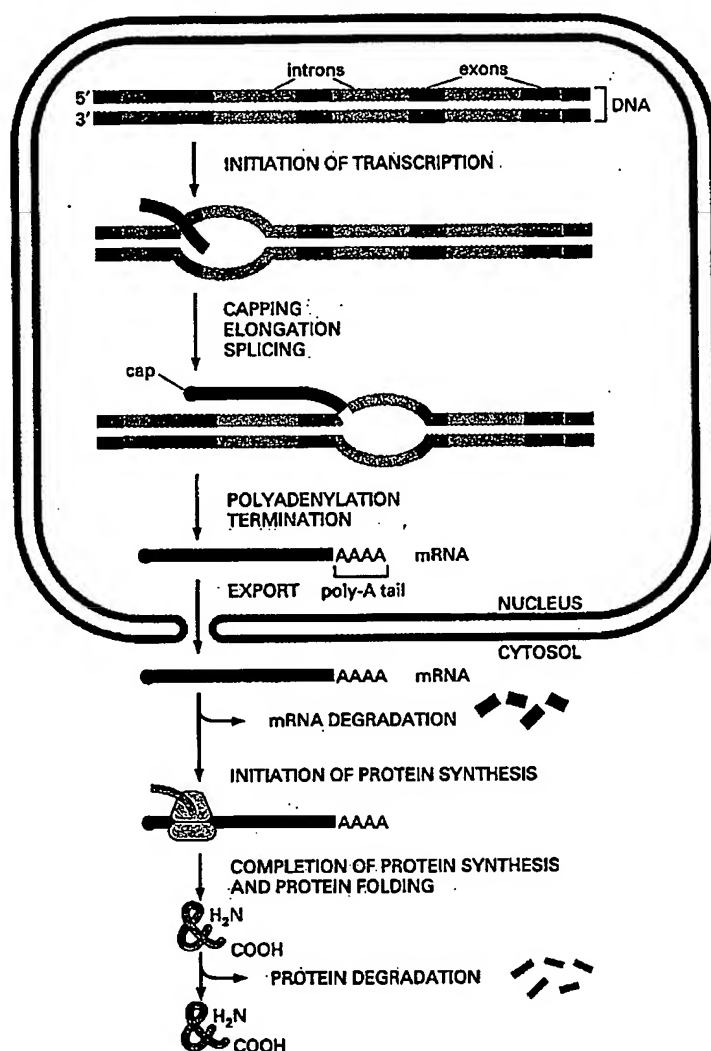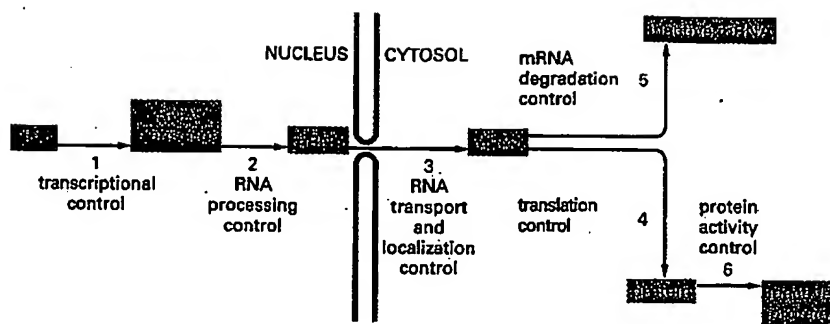
Figure 6–90 The production of a protein by a eucaryotic cell. The final level of each protein in a eucaryotic cell depends upon the efficiency of each step depicted.

ure 6–90) could be regulated by the cell for each individual protein. However, as we shall see in Chapter 7, the initiation of transcription is the most common point for a cell to regulate the expression of each of its genes. This makes sense, inasmuch as the most efficient way to keep a gene from being expressed is to block the very first step—the transcription of its DNA sequence into an RNA molecule.

Summary

The translation of the nucleotide sequence of an mRNA molecule into protein takes place in the cytoplasm on a large ribonucleoprotein assembly called a ribosome. The amino acids used for protein synthesis are first attached to a family of tRNA molecules, each of which recognizes, by complementary base-pair interactions, particular sets of three nucleotides in the mRNA (codons). The sequence of nucleotides in the mRNA is then read from one end to the other in sets of three according to the genetic code.

To initiate translation, a small ribosomal subunit binds to the mRNA molecule at a start codon (AUG) that is recognized by a unique initiator tRNA molecule. A large ribosomal subunit binds to complete the ribosome and begin the elongation phase of protein synthesis. During this phase, aminoacyl tRNAs—each bearing a specific amino acid bind sequentially to the appropriate codon in mRNA by forming complementary base pairs with the tRNA anticodon. Each amino acid is added to the C-terminal end of the growing polypeptide by means of a cycle of three sequential

NUCLEUS | CYTOSOL

1 transcriptional control

2 RNA processing control

3 RNA transport and localization control

mRNA degradation control 5

translation control 4

protein activity control 6

Figure 7–5 Six steps at which eucaryotic gene expression can be controlled. Controls that operate at steps 1 through 5 are discussed in this chapter. Step 6, the regulation of protein activity, includes reversible activation or inactivation by protein phosphorylation (discussed in Chapter 3) as well as irreversible inactivation by proteolytic degradation (discussed in Chapter 6).

Gene Expression Can Be Regulated at Many of the Steps in the Pathway from DNA to RNA to Protein

If differences among the various cell types of an organism depend on the particular genes that the cells express, at what level is the control of gene expression exercised? As we saw in the last chapter, there are many steps in the pathway leading from DNA to protein, and all of them can in principle be regulated. Thus a cell can control the proteins it makes by (1) controlling when and how often a given gene is transcribed (**transcriptional control**), (2) controlling how the RNA transcript is spliced or otherwise processed (**RNA processing control**), (3) selecting which completed mRNAs in the cell nucleus are exported to the cytosol and determining where in the cytosol they are localized (**RNA transport and localization control**), (4) selecting which mRNAs in the cytoplasm are translated by ribosomes (**translational control**), (5) selectively destabilizing certain mRNA molecules in the cytoplasm (**mRNA degradation control**), or (6) selectively activating, inactivating, degrading, or compartmentalizing specific protein molecules after they have been made (**protein activity control**) (Figure 7–5).

For most genes transcriptional controls are paramount. This makes sense because, of all the possible control points illustrated in Figure 7–5, only transcriptional control ensures that the cell will not synthesize superfluous intermediates. In the following sections we discuss the DNA and protein components that perform this function by regulating the initiation of gene transcription. We shall return at the end of the chapter to the additional ways of regulating gene expression.

Summary

The genome of a cell contains in its DNA sequence the information to make many thousands of different protein and RNA molecules. A cell typically expresses only a fraction of its genes, and the different types of cells in multicellular organisms arise because different sets of genes are expressed. Moreover, cells can change the pattern of genes they express in response to changes in their environment, such as signals from other cells. Although all of the steps involved in expressing a gene can in principle be regulated, for most genes the initiation of RNA transcription is the most important point of control.

DNA-BINDING MOTIFS IN GENE REGULATORY PROTEINS

How does a cell determine which of its thousands of genes to transcribe? As mentioned briefly in Chapters 4 and 6, the transcription of each gene is controlled by a regulatory region of DNA relatively near the site where transcription begins. Some regulatory regions are simple and act as switches that are thrown by a single signal. Many others are complex and act as tiny microprocessors, responding to a variety of signals that they interpret and integrate to switch the neighboring gene on or off. Whether complex or simple, these switching devices

occur in the germ line, the cell lineage that gives rise to sperm or eggs. Most of the DNA in vertebrate germ cells is inactive and highly methylated. Over long periods of evolutionary time, the methylated CG sequences in these inactive regions have presumably been lost through spontaneous deamination events that were not properly repaired. However promoters of genes that remain active in the germ cell lineages (including most housekeeping genes) are kept unmethylated, and therefore spontaneous deaminations of Cs that occur within them can be accurately repaired. Such regions are preserved in modern day vertebrate cells as CG islands. In addition, any mutation of a CG sequence in the genome that destroyed the function or regulation of a gene in the adult would be selected against, and some CG islands are simply the result of a higher than normal density of critical CG sequences.

The mammalian genome contains an estimated 20,000 CG islands. Most of the islands mark the 5' ends of transcription units and thus, presumably, of genes. The presence of CG islands often provides a convenient way of identifying genes in the DNA sequences of vertebrate genomes.
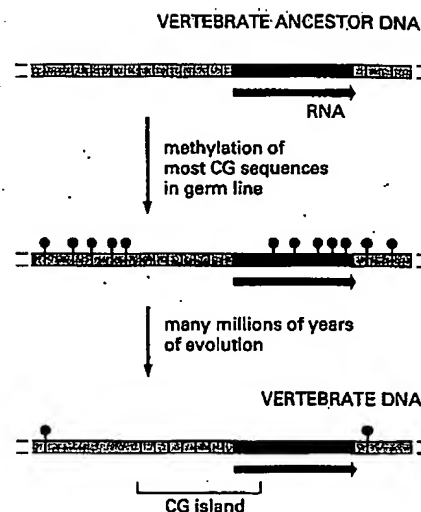
Summary

The many types of cells in animals and plants are created largely through mechanisms that cause different genes to be transcribed in different cells. Since many specialized animal cells can maintain their unique character through many cell division cycles and even when grown in culture, the gene regulatory mechanisms involved in creating them must be stable once established and heritable when the cell divides. These features endow the cell with a memory of its developmental history. Bacteria and yeasts provide unusually accessible model systems in which to study gene regulatory mechanisms. One such mechanism involves a competitive interaction between two gene regulatory proteins, each of which inhibits the synthesis of the other; this can create a flip-flop switch that switches a cell between two alternative patterns of gene expression. Direct or indirect positive feedback loops, which enable gene regulatory proteins to perpetuate their own synthesis, provide a general mechanism for cell memory. Negative feedback loops with programmed delays form the basis for cellular clocks.

In eucaryotes the transcription of a gene is generally controlled by combinations of gene regulatory proteins. It is thought that each type of cell in a higher eucaryotic organism contains a specific combination of gene regulatory proteins that ensures the expression of only those genes appropriate to that type of cell. A given gene regulatory protein may be active in a variety of circumstances and typically is involved in the regulation of many genes.

In addition to diffusible gene regulatory proteins, inherited states of chromatin condensation are also used by eucaryotic cells to regulate gene expression. An especially dramatic case is the inactivation of an entire X chromosome in female mammals. In vertebrates DNA methylation also functions in gene regulation, being used mainly as a device to reinforce decisions about gene expression that are made initially by other mechanisms. DNA methylation also underlies the phenomenon of genomic imprinting in mammals, in which the expression of a gene depends on whether it was inherited from the mother or the father.

POSTTRANSCRIPTIONAL CONTROLS

In principle, every step required for the process of gene expression could be controlled. Indeed, one can find examples of each type of regulation, although any one gene is likely to use only a few of them. Controls on the initiation of gene transcription are the predominant form of regulation for most genes. But other controls can act later in the pathway from DNA to protein to modulate the amount of gene product that is made. Although these **posttranscriptional controls**, which operate after RNA polymerase has bound to the gene's promoter and begun RNA synthesis, are less common than *transcriptional control*, for many genes they are crucial.

VERTEBRATE ANCESTOR DNA

RNA

methylation of most CG sequences in germ line

many millions of years of evolution

VERTEBRATE DNA

CG island

Figure 7–86 A mechanism to explain both the marked overall deficiency of CG sequences and their clustering into CG islands in vertebrate genomes. A *black line* marks the location of a CG dinucleotide in the DNA sequence, while a *red* "lollipop" indicates the presence of a methyl group on the CG dinucleotide. CG sequences that lie in regulatory sequences of genes that are transcribed in germ cells are unmethylated and therefore tend to be retained in evolution. Methylated CG sequences, on the other hand, tend to be lost through deamination of 5-methyl C to T, unless the CG sequence is critical for survival.

Discordant Protein and mRNA Expression in Lung Adenocarcinomas*

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The relationship between gene expression measured at the mRNA level and the corresponding protein level is not well characterized in human cancer. In this study, we compared mRNA and protein expression for a cohort of genes in the same lung adenocarcinomas. The abundance of 165 protein spots representing 98 individual genes was analyzed in 76 lung adenocarcinomas and nine non-neoplastic lung tissues using two-dimensional polyacrylamide gel electrophoresis. Specific polypeptides were identified using matrix-assisted laser desorption/ionization mass spectrometry. For the same 85 samples, mRNA levels were determined using oligonucleotide microarrays, allowing a comparative analysis of mRNA and protein expression among the 165 protein spots. Twenty-eight of the 165 protein spots (17%) or 21 of 98 genes (21.4%) had a statistically significant correlation between protein and mRNA expression ($r > 0.2445$; $p < 0.05$); however, among all 165 proteins the correlation coefficient values (r) ranged from -0.467 to 0.442. Correlation coefficient values were not related to protein abundance. Further, no significant correlation between mRNA and protein expression was found ($r = -0.025$) if the average levels of mRNA or protein among all samples were applied across the 165 protein spots (98 genes). The mRNA/protein correlation coefficient also varied among proteins with multiple isoforms, indicating potentially separate isoform-specific mechanisms for the regulation of protein abundance. Among the 21 genes with a significant correlation between mRNA and protein, five genes differed significantly between stage I and stage III lung adenocarcinomas. Using a quantitative analysis of mRNA and protein expression within the same lung adenocarcinomas, we showed that only a subset of the proteins exhibited a significant correlation with mRNA abundance. *Molecular & Cellular Proteomics 1:304–313, 2002.*

Lung cancer is the leading cause of cancer death for both men and women in the United States. Adenocarcinomas of the lung comprise ~40% of all new cases of non-small cell

lung cancer and are now the most common histologic type. Functional genomics, broadly defined as the comprehensive analysis of genes and their products, have become a recent focus of the life sciences (1). Application of these approaches to lung adenocarcinomas has the potential to aid in the identification of high risk patients with resectable early stage lung cancer that may benefit from adjuvant therapy, as well as to identify new therapeutic targets. In human lung cancer, however, little is currently understood regarding the relationship between gene expression as determined by measuring mRNA levels and the corresponding abundance of the protein products.

A number of powerful techniques for analysis of gene expression have been used including differential display (2), serial analysis of gene expression (3), DNA microarrays (4), and proteomics via two-dimensional polyacrylamide gel electrophoresis and mass spectrometry (5). Bioinformatics tools have also been developed to help determine quantitative mRNA/protein expression profiles of all types of cells and tissues (6) and now can be applied to benign and malignant tumors. DNA microarrays (cDNA and oligonucleotide) permit the parallel assessment of thousands of genes and have been utilized in gene expression monitoring (7), polymorphism analysis (8), and DNA sequencing (9). Recent studies have focused on classification or identification of subgroups of lung tumors using DNA microarrays (10, 11). The use of mRNA expression patterns by themselves, however, is insufficient for understanding the expression of protein products, as additional post-transcriptional mechanisms, including protein translation, post-translational modification, and degradation, may influence the level of a protein present in a given cell or tissue. Proteomic analyses, a complementary technology to DNA microarrays for monitoring gene expression, involves protein separation and quantitative assessment of protein spots using 2D[1]-PAGE and protein identification using mass spectrometry. By combining proteomic and transcriptional analyses of the same samples, however, it may be possible to understand the complex mechanisms influencing protein expression in human cancer.

In this study, we determined mRNA and protein levels for 165 proteins (98 genes) in 76 lung adenocarcinomas and nine

[1] The abbreviations used are: 2D, two-dimensional; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry.

TABLE I
Correlation coefficients of protein and mRNA where only one spot was present on 2D gels

r, correlation coefficient value > 0.2445; $p < 0.05$. Values in boldface are significant at $p < 0.05$.

Spot	Unigene	Gene name	r	Protein name
1104	Hs.184510	SFN	0.4337	14-3-3 σ
0994	Hs.77840	ANXA4	0.4219	Annexin IV
1314	Hs.10958	DJ-1	0.3982	DJ-1 protein/MER5
1454	Hs.75428	SOD1	0.3863	Superoxide dismutase (Cu-Zn)
1638	Hs.227751	LGALS1	0.3318	Galectin 1
0264	Hs.129548	HNRPK	0.3034	Transformation up-regulated nuclear protein
1405	Hs.111334	FTL	0.2849	Ferritin light chain
0863	Hs.300711	ANXA5	0.2468	Annexin V
1252	Hs.4745	PSMC	0.2445	26 S proteasome p28
0906	Hs.234489	LDHB	0.4420	L-lactate dehydrogenase H chain (LDH-B)
1171	Hs.241515	COX11	0.2310	COX 11
1160	Hs.181013	PGAM1	0.2023	Phosphoglycerate mutase
0759	Hs.74635	DLD	0.1985	Dihydrolipoamide dehydrogenase precursor
1193	Hs.83383	AOE372	0.1932	Antioxidant enzyme AOE372
0172	Hs.3069	HSPA9B	0.1872	GRP75
0777	Hs.979	PDHB	0.1855	Pyruvate dehydrogenase E1-β subunit precursor
1249	Hs.226795	GSTP1	0.1773	Glutathione S-transferase pi (GST-pi)
1685	Hs.76136	TXN	0.1732	Thioredoxin
1205	Hs.82314	HPRT1	0.1588	HG phosphoribosyltransferase
1230	Hs.279860	TPT1	0.1466	Translationally controlled tumor protein (TCTP)
0603	Hs.181357	LAMR1	0.1463	LAMR
1358	Hs.28914	APRT	0.1399	Adenine phosphoribosyl transferase
1410	Hs.82113	DUT	0.1213	dUTP pyrophosphatase (dUTPase)
1825	Hs.112378	LIMS1	0.1213	Pinch-2 protein
0871	Hs.250502	CA8	0.1122	Carbonic anhydrase-related protein; Syntaxin
0289	Hs.82916	CCT6A	0.1106	Chaperonin-like protein
1143	Hs.11465	GSTTLp28	0.0997	Glutathione S-transferase homolog (GST homolog)
1456	Hs.118638	NME1	0.0932	Nm23 (NDPKA)
1598	Hs.278503	RIIG	0.0905	RIIG (U32331)
1354	Hs.89761	ATP5D	0.0904	F1F0-type ATP synthase subunit d
1445	Hs.155485	HIP2	0.0843	Huntingtin interacting protein 2 (HIP2)
1479	Hs.177486	APP	0.0746	Amyloid B4A
0608	Hs.182285	KRT19	0.0439	Cytokeratin 19
1071	Hs.10842	RAN	0.0277	GTP-binding nuclear protein RAN(TC4)
0991	Hs.297939	CTSB	0.0264	Cathepsin B
0842	Hs.77274	PLAU	0.0248	Urokinase plasminogen activator
0823	Hs.198248	B4GALT1	0.0183	β 1,4-galactosyl transferase
0613	Hs.1247	APOA4	0.0176	Apolipoprotein A4 (ApoA4)
1338	Hs.104143	CLTA	0.0123	Clathrin light chain A
0902	Hs.5123	SID6-306	0.0117	Cytosolic inorganic pyrophosphatase
1688	Hs.1473	GRP	−0.0040	Preprogastrin-releasing peptide
0265	Hs.274402	HSPA1B	−0.0071	Heat shock-induced protein
1414	Hs.77541	ARF5	−0.0096	ADP-ribosylation factor 1
0710	Hs.97206	HIP1	−0.0114	Huntingtin interacting protein 1 (HIP1)
0532	Hs.170328	MSN	−0.0132	Moesin/E
0525	Hs.284255	ALPP	−0.0148	Alkaline phosphate, placental
0513	Hs.76901	PDIR	−0.0289	Protein disulfide isomerase-related protein 5
1659	Hs.256697	HINT	−0.0312	Protein kinase C inhibitor
1262	Hs.7016	RAB7	−0.0362	Rab 7 protein
0190	Hs.184411	ALB	−0.0470	Albumin
0948	Hs.2795	LDHA	−0.0549	Lactate dehydrogenase-A (LDHA)
0502	Hs.180532	GPI	−0.0575	Hsp89
0152	Hs.75410	HSPA5	−0.0640	GRP78
1054	Hs.74276	CLIC1	−0.0686	Nuclear chloride channel (RNCC protein)
0709	Hs.253495	SFTPD	−0.0936	Pulmonary surfactant protein D
0867	Hs.78998	PCNA	−0.0982	PCNA
0165	Hs.180414	HSPA8	−0.1014	Heat shock cognate protein, 71 kDa
1109	Hs.75103	YWHAZ	−0.1018	14-3-3 ζ/δ
0137	Hs.554	SSA2	−0.1032	Ro/ss-A antigen

TABLE I—continued

Spot	Unigene	Gene name	r	Protein name
0278	Hs.4112	TCP1	−0.1237	T-complex protein I, α subunit
1769	Hs.9614	NPM1	−0.1738	B23/numatrin
0089	Hs.74335	HSPCB	−0.2049	Hsp90
2511	Hs.153179	FABP5	−0.2109	E-FABP/FABP5
1739	Hs.16488	CALR	−0.2344	Calreticulin 32
1138	Hs.301961	GSTM4	−0.2438	Glutathione S-transferase M4 (GST m4)
2533	Hs.77060	PSMB6	−0.2512	Macropain subunit Δ

non-neoplastic lung tissues. Protein levels were determined using quantitative 2D-PAGE analysis, and the separated protein polypeptides were identified using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The corresponding mRNA levels for the identified proteins within the same samples were determined using oligonucleotide microarrays. Correlation analyses showed that protein abundance is likely a reflection of the transcription for a subset of proteins, but translation and post-translational modifications also appear to influence the expression levels of many individual proteins in lung adenocarcinomas.

EXPERIMENTAL PROCEDURES

Tissues—Fifty-seven stage I and 19 stage III lung adenocarcinomas, as well as nine non-neoplastic lung tissue samples, were used for protein and mRNA analyses. Patient consent was obtained, and the project was approved by the Institutional Review Board. All tissues were obtained after resection at the University of Michigan Health System between May 1991 and July 1998. Tissues were all snap-frozen in liquid nitrogen and then stored at −80 °C. The patients included 46 females and 30 males ranging in age from 40.9 to 84.6 (average 63.8) years. Most patients (66/76) demonstrated a positive smoking history. Sixty-one tumor samples were classified as bronchial-derived, 14 were classified as bronchoalveolar, and one had both features. Eighteen tumor samples were classified as well differentiated, 38 were classified as moderate, and 19 were classified as poorly differentiated adenocarcinomas. Hematoxylin-stained cryostat sections (5 μm), prepared from the same tumor pieces to be utilized for protein and mRNA isolation, were evaluated by a pathologist and compared with hematoxylin- and eosin-stained sections made from paraffin blocks of the same tumors. Specimens were excluded from analysis if they showed unclear or mixed histology (e.g. adenosquamous), tumor cellularity less than 70%, potential metastatic origin as indicated by previous tumor history, extensive lymphocytic infiltration, or fibrosis or if the patient had received prior chemotherapy or radiotherapy.

Oligonucleotide Array Hybridization—The HuGeneFL oligonucleotide arrays (Affymetrix, Santa Clara, CA) containing 6800 genes were used in this study. Total RNA was isolated from all samples using Trizol reagent (Invitrogen). The resulting RNA was then subjected to further purification using RNeasy spin columns (Qiagen). Preparation of cRNA, hybridization, and scanning of the HuGeneFL arrays were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). Data analysis was performed using GeneChip 4.0 software. The gene expression profile of each tumor was normalized to the median gene expression profile for the entire sample. Details of data trimming and normalization are described elsewhere (11).

2D-PAGE and Quantitative Protein Analysis—Tissue for both protein and mRNA isolation came from contiguous areas of each sample. Protein separation using 2D-PAGE, silver staining, and digitization

were performed as described previously (12, 13). Our 2D-PAGE system allows us to run 20 gels at one time (one batch). Spot detection and quantification were accomplished utilizing Bio Image Visage System software (Bioimage Corp., Ann Arbor, MI). The integrated intensity of each spot was calculated as the measured optical density units × mm². Of the total possible 2000 spots detectable on each gel, 820 spots on the gel of each sample were matched using a Gel-ed match program with the same spots on a chosen "master" gel. In each sample, 250 ubiquitously expressed reference spots were used to adjust for variations between gels, such as that created by subtle differences in protein loading or gel staining. Slight differences because of batch were corrected after spot-size quantification.

Mass Spectrometry and 2D Western Blotting—Preparative 2D gels were run using extracts from A549 lung adenocarcinoma cells (obtained from ATCC) and using the identical experimental conditions as the analytical 2D gels, except 30% more protein was loaded. The resolved protein gels were silver-stained using successive incubations in 0.02% sodium thiosulfate for 2 min, 0.1% silver nitrate for 40 min, and 0.014% formaldehyde plus 2% sodium carbonate for 10 min. For protein identification, protein polypeptides underwent trypsin digestion followed by MALDI-MS using a MALDI-TOF Voyager-DE mass spectrometer (Perseptive Biosystems, Framingham, MA). The masses were compared with known trypsin digest databases using the MS-FIT database (University of California, San Francisco; prospector.uosf.edu/ucsfhtml3.2/msfit.htm). Some of the polypeptides included in the analysis had been identified prior to this study on the basis of sequencing (14). The identified protein spots used in this paper are shown in Fig. 1A. The method for 2D-PAGE Western blot verification was as described previously (15). The 2D Western blots of GRP58 and Op18 are shown in Fig. 1, C and E; the others, such as GRP78, GRP75, HSP70, HSC70, KRT8, KRT18, KRT19, Vimentin, ApoJ, 14-3-3, Annexin I, Annexin II, PGP9.5, DJ-1, GST-pi, and PGAM, are described elsewhere.[2]

Statistical Analysis—Missing values were replaced with the mean value of the protein spot. The transform x → log (1 + x) was applied to normalize all protein expression values. The relationship between protein and mRNA expression levels within the same samples was examined using the Spearman correlation coefficient analysis (16). To identify potentially significant correlations between gene and protein expression, we used an analytical strategy similar to SAM (significance analysis of microarrays) (17), which uses a permutation technique to determine the significance of changes in gene expression between different biological states. To obtain permuted correlation coefficients between gene and protein expression, genes were exchanged first in such a way that permutated correlation coefficient were calculated based on pseudo pairs of genes and proteins. The distribution of permutated correlation coefficients became stable after 60 permutations. This procedure was then repeated 60 times to obtain 60 sets of permutated correlation coefficients. For each of the 60 permutations, the correlations of genes and proteins were ranked

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[2] Chen *et al.*, submitted for publication.

TABLE II

Correlation coefficients of protein and mRNA where multiple isoforms were present on 2D gels

r, correlation coefficient value > 0.2445; $p < 0.05$. Values in boldface are significant at $p < 0.05$.

Spot	Unigene	Gene name	r	Protein name
1494	Hs.81915	LAP18	0.4003	OP18 (Stathmin)
0957	Hs.77899	TPM1	0.3930	Tropomyosins 1-5
0353	Hs.289101	GRP58	0.3802	Protease disulfide isomerase (GRP58)
0855	Hs.169476	GAPD	0.3693	Glyceraldehyde-3-phosphate dehydrogenase
1198	Hs.41707	HSPB3	0.3668	Hsp27
1203	Hs.83848	TPI1	0.3395	Triose phosphate isomerase (TPI)
0523	Hs.65114	KRT18	0.3335	Cytokeratin 18
1492	Hs.81915	LAP18	0.3234	OP18 (Stathmin)
1493	Hs.81915	LAP18	0.3154	OP18 (Stathmin)
1181	Hs.78225	ANXA1	0.3102	Annexin variant I
0439	Hs.242463	KRT8	0.3049	Cytokeratin 8
0505	Hs.297753	VIM	0.2839	Vimentin
0593	Hs.297753	VIM	0.2809	Vimentin
1874	Hs.75313	AKR1B1	0.2780	Aldose reductase
0935	Hs.75544	YWHAH	0.2775	14-3-3 η
2524	Hs.78225	ANXA1	0.2612	Annexin I
2324	Hs.65114	KRT18	0.2601	Cytokeratin 18
1192	Hs.41707	HSPB3	0.2558	Hsp27
0350	Hs.289101	GRP58	0.2516	Phospholipase C (GRP58)
0992	Hs.75313	AKR1B1	−0.2460	Aldose reductase
0861	Hs.75313	AKR1B1	0.0761	Aldose reductase
0853	Hs.75313	AKR1B1	−0.0875	Aldose reductase
2503	Hs.76392	ALDH1	−0.0565	Aldehyde dehydrogenase
0381	Hs.76392	ALDH1	−0.0371	Aldehyde dehydrogenase
0371	Hs.76392	ALDH1	−0.0880	Aldehyde dehydrogenase
1179	Hs.78225	ANXA1	0.2052	Annexin variant I
0762	Hs.78225	ANXA1	−0.0739	Annexin I
0760	Hs.78225	ANXA1	−0.0228	Annexin I
2506	Hs.217493	ANXA2	0.2223	Lipocotin (annexin II)
0772	Hs.217493	ANXA2	0.2080	Lipocotin (annexin II)
0723	Hs.217493	ANXA2	0.0701	Lipocotin
1239	Hs.93194	APOA1	0.1133	Apolipoprotein A1 (ApoA1)
1237	Hs.93194	APOA1	−0.0373	Apolipoprotein A1 (ApoA1)
1234	Hs.93194	APOA1	−0.0894	Apolipoprotein A1 (ApoA1)
0428	Hs.25	ATP5B	0.0080	ATP synthase β subunit precursor
0427	Hs.25	ATP5B	0.0122	ATP synthase β subunit precursor
0424	Hs.25	ATP5B	−0.0992	ATP synthase β subunit precursor
0883	Hs.75106	CLU	−0.0483	Apolipoprotein J (ApoJ)
0780	Hs.75106	CLU	−0.0443	Apolipoprotein J (ApoJ)
1527	Hs.119140	EIF5A	−0.0726	eIF-5A
1484	Hs.119140	EIF5A	−0.0376	eIF-5A
1728	Hs.5241	FABP1	−0.1916	L-FABP
1712	Hs.5241	FABP1	−0.0473	L-FABP
0947	Hs.169476	GAPD	0.1745	Glyceraldehyde-3-phosphate dehydrogenase
1232	Hs.75207	GLO1	0.2249	Glyoxalase-I
1229	Hs.75207	GLO1	0.0450	Glyoxalase-1
1595	Hs.158300	HAP1	−0.0137	Huntingtin-associated protein 1 (neuroan 1)
1810	Hs.75990	HP	−0.4672	α-Haptoglobin
1459	Hs.75990	HP	0.0802	α-Haptoglobin
1458	Hs.75990	HP	−0.0305	α-Haptoglobin
0619	Hs.75990	HP	0.0461	B-haptoglobin
0615	Hs.75990	HP	−0.0034	B-haptoglobin
1250	Hs.41707	HSPB3	−0.1024	Hsp27
0549	Hs.79037	HSPD1	0.1074	Hsp60
0338	Hs.79037	HSPD1	0.2285	Hsp60
0333	Hs.79037	HSPD1	0.1383	Hsp60
0331	Hs.79037	HSPD1	0.1603	Hsp60
2381	Hs.65114	KRT18	0.2016	Cytokeratin 18
0535	Hs.65114	KRT18	0.1106	Cytokeratin 18

TABLE II—continued.
Correlation coefficients of protein and mRNA where multiple isoforms were present on 2D gels
r, correlation coefficient value > 0.2445; $p < 0.05$. Values in boldface are significant at $p < 0.05$.

Spot	Unigene	Gene name	r	Protein name
0529	Hs.65114	KRT18	0.1279	Cytokeratin 18
0528	Hs.65114	KRT18	0.0414	Cytokeratin 18
0527	Hs.65114	KRT18	0.0436	Cytokeratin 18
0514	Hs.65114	KRT18	0.0733	Cytokeratin 18
0451	Hs.242463	KRT8	−0.0111	Cytokeratin 8
0446	Hs.242463	KRT8	0.0347	Cytokeratin 8
0444	Hs.242463	KRT8	−0.1311	Cytokeratin 8
0443	Hs.242463	KRT8	0.0942	Cytokeratin 8
1488	Hs.81915	LAP18	0.0495	OP18 (Stathmin)
0321	Hs.75655	P4HB	−0.0546	PDI (proly-4-OH-B)
0320	Hs.75655	P4HB	−0.0041	PDI (proly-4-OH-B)
1063	Hs.75323	PHB	0.0441	Prohibitin
0837	Hs.75323	PHB	0.1402	Prohibitin
0326	Hs.297681	SERPINA1	−0.0227	α-1-Antitripsin
0322	Hs.297681	SERPINA1	−0.0277	α-1-Antitripsin
0241	Hs.297681	SERPINA1	−0.0148	α-1-Antitripsin
1280	Hs.301254	SFTPA1	−0.1488	Pulmonary surfactant-associated protein
1278	Hs.301254	SFTPA1	−0.2040	Pulmonary surfactant-associated protein
0866	Hs.73980	TNNT1	0.1162	Troponin T
0778	Hs.73980	TNNT1	0.0740	Troponin T
1213	Hs.83848	TPI1	0.0024	Triose phosphate isomerase (TPI)
1210	Hs.83848	TPI1	0.0490	Triose phosphate isomerase (TPI)
1207	Hs.83848	TPI1	−0.1615	Triose phosphate isomerase (TPI)
1204	Hs.83848	TPI1	0.0209	Triose phosphate isomerase (TPI)
1202	Hs.83848	TPI1	0.0721	Triose phosphate isomerase (TPI)
1161	Hs.83848	TPI1	0.2265	Triose phosphate isomerase (TPI)
1052	Hs.77899	TPM1	−0.1040	Tropomysin clean-product
1039	Hs.77899	TPM1	−0.2899	Cytoskeletal tropomyosin
1035	Hs.77899	TPM1	−0.3821	Tropomyosin
0783	Hs.77899	TPM1	0.0757	Tropomyosins 1-5
1574	Hs.194366	TTR	−0.0065	Transthyretin
0809	Hs.194366	TTR	0.0399	Transthyretin multimere
2202	Hs.76118	UCHL1	−0.0220	Ubiquitin carboxyl-terminal hydrolase isozyme L1
1246	Hs.76118	UCHL1	−0.1261	Ubiquitin carboxyl-terminal hydrolase isozyme L1
1242	Hs.76118	UCHL1	0.1473	Ubiquitin carboxyl-terminal hydrolase isozyme L1
0606	Hs.297753	VIM	0.0951	Vimentin
0594	Hs.297753	VIM	−0.2684	Vimentin-derived protein (vid4)
0508	Hs.297753	VIM	0.1008	Vimentin-derived protein (vid2)
0419	Hs.297753	VIM	0.0032	Vimentin-derived protein (vid1)
1279	Hs.75544	YWHAH	0.0059	14-3-3 η

such that $p_p(l)$ denotes the lth largest correlation coefficient for pth permutation. Hence, the expected correlation coefficient, $p_E(l)$, was the average over the 60 permutations, $p_E(l) = \Sigma^{60}_{p=1} p_p(l)/60$. A scatter plot of observed correlations ($p(l)$) versus the expected correlations is shown in Fig. 2D. For this study, we chose threshold $\Delta = 0.115$ so that correlation would be considered significant if absolute value of difference between $p(l)$ and $p_E(l)$ was greater than the threshold. Twenty-nine (including one with observed correlation coefficient −0.4672) of 165 pairs of gene and protein expression were called significant in such criteria, and the permuted data generated an average of 5.1 falsely significant pairs of gene and protein expression. This provided an estimated false discovery rate (the percentage of pairs of gene and protein expression identified by chance) for our data set.

RESULTS

Correlation of Individual Proteins and mRNA Expression within Each Tumor—We have examined quantitatively 165 protein spots on 2D gels representing 98 genes and compared protein levels with mRNA levels for a cohort of 85 lung adenocarcinomas and normal lung samples. Of the 165 protein spots, 69 proteins were represented by only one known spot on 2D gels for an individual gene, whereas 96 protein spots showed multiple protein products from 29 different genes. 2D Western blotting verified the proteins identified by mass spectrometry when specific antibodies were available. Spearman correlation coefficients of the proteins and their associated mRNA for each protein spot were generated using all 76 lung adenocarcinomas and nine non-neoplastic lung tissues (see Tables I and II, and see Figs. 1 and 2). The correlation coefficients (r) ranged from −0.467 to 0.442 (Fig. 2D). A total of 28 protein spots (21 genes) were found to have a statistically significant correlation between expression of

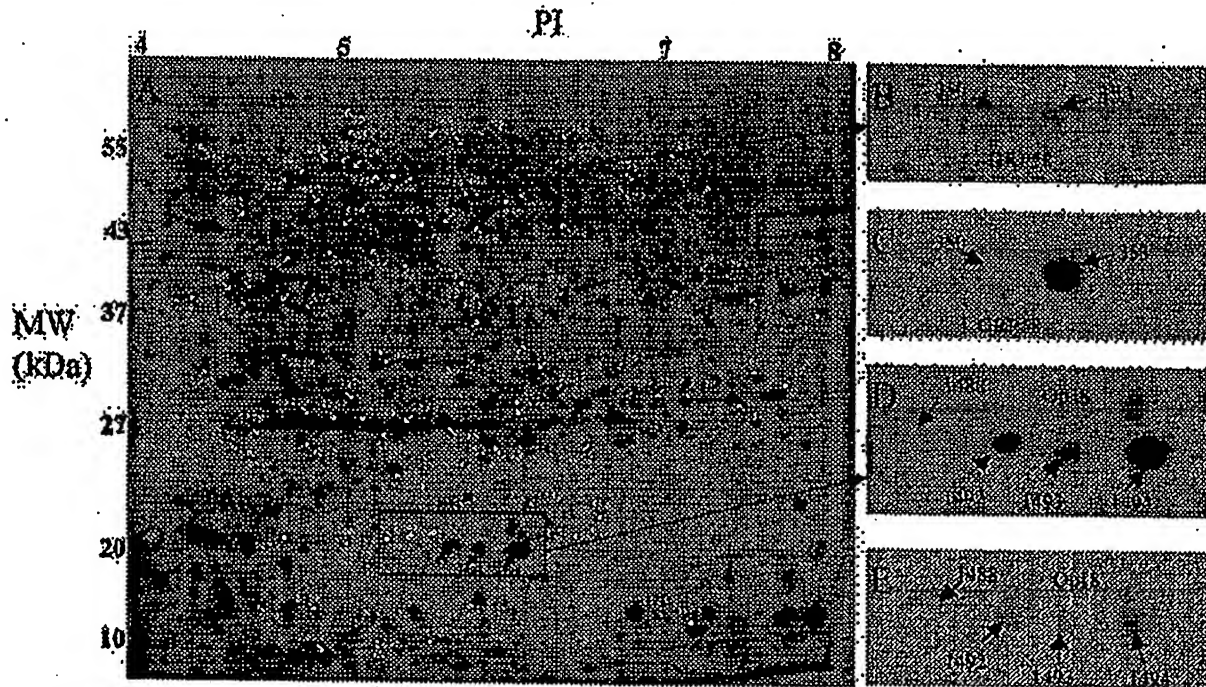Fig. 1. *A*, digital image of a silver-stained 2D-PAGE separation of a stage I lung adenocarcinoma showing protein spots separated by molecular mass *(MW)* and isoelectric point *(PI)*. Twenty-eight protein spots whose expression levels are correlated with mRNA abundance are indicated by the *black arrows*. *B*, the outlined areas of *A* showing protein GRP58. *C*, 2D Western blot of GRP58 from the A549 lung adenocarcinoma cell line. *D*, the outlined areas of *A* showing the protein isoforms of Op18. *E*, 2D Western blot of Op18 from A549 cells.

their protein and mRNA ($r > 0.2445; p < 0.05$). This accounts for 17% (28/165) of the 165 protein spots. Among the 69 genes for which only a single protein spot was known (Table I), nine genes (9/69, 13%) were observed to show a statistically significant relationship between protein and mRNA abundance ($r > 0.2445; p < 0.05$). The proteins whose expression levels were correlated with their mRNA abundance included those involved in signal transduction, carbohydrate metabolism, apoptosis, protein post-translational modification, structural proteins, and heat shock proteins (Table III).

Individual Isoforms of the Same Protein Have Different Protein/mRNA Correlation Coefficients—Of the 165 protein spots, 96 represent protein products of 29 genes with at least two isoforms. Among these 96 protein spots, 19 (19/96 protein spots, 20%) showed a statistically significant correlation between their protein and mRNA expression ($r > 0.2445; p < 0.05$) (Table II) and represented 12 genes (12/29, 41%). Individual isoforms of the same protein demonstrated different protein/mRNA correlation coefficients. For example, 2D-PAGE/Western analysis revealed four isoforms of OP18 differing in regards to isoelectric point but similar in molecular weight. Three of the four isoforms (spots 1492, 1493, and 1494) showed a statistically significant correlation between their protein and mRNA abundance ($r = 0.3234, 0.3154$, and 0.4003, respectively). The forth isoform (spot 1488) showed no correlation be-

tween protein and mRNA expression ($r = 0.0495$). Similarly, just one of five quantified isoforms of cytokeratin 8 (spot 439) demonstrated a statistically significant correlation between protein and mRNA abundance ($r = 0.3049; p < 0.05$) (Table II).

In addition to differences in the relationship between mRNA levels and protein expression among separate isoforms, some genes with very comparable mRNA levels showed a 24-fold difference in their protein expression. Genes with comparable protein expression levels also showed up to a 28-fold variance in their mRNA levels.

Lack of Correlation for mRNA and Protein Expression when Using Average Tumor Values across All 165 Protein Spots (98 Genes)—The relationship between mRNA and protein expression was also examined by using the average expression values for all samples. To analyze this relationship using this approach, the average value for each protein or mRNA was generated using all 85 lung tissue samples. The range of normalized average protein values ranged from −0.0846 to 0.0979 (raw value 0.0036 to 4.1947), and the range for mRNA was from 0 to 15260.5 for all 165 individual protein spots. The Spearman correlation coefficient for the whole data set (165 protein spots/98 genes) was −0.025 (Fig. 3A). Even for the 28 protein spots (Fig. 2D) that were found to have a statistically significant correlation between their mRNA and protein, use of
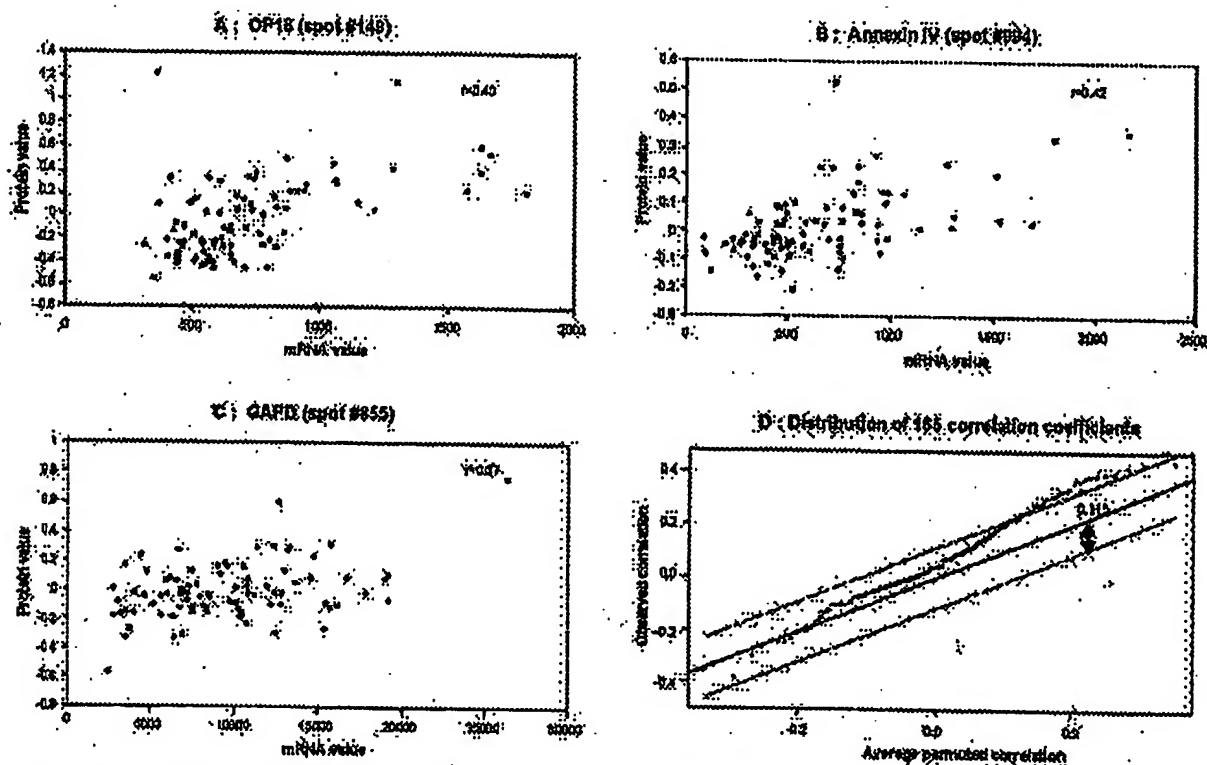
Fig. 2. A–C, plots showing the correlation between mRNA and protein for the three selected genes Op18, Annexin IV, and GAPD for all 76 lung adenocarcinomas and nine non-neoplastic lung samples ($p < 0.05$). D, distribution of all 165 Spearman correlation coefficients (r) and verification analysis using SAM. A more detailed description of the method is provided under "Experimental Procedures." Approximately 17% of the 165 proteins demonstrate a significant correlation between mRNA and protein levels as demonstrated by the values shown beyond the outer range of threshold $\Delta = 0.115$. Normalized protein values were used, thus negative values for some proteins are observed.

the average value resulted in a correlation coefficient value of -0.035, which was not significant (Fig. 3B).

Lack of a Relationship between Protein/mRNA Correlation Coefficients and Average Protein Abundance—To determine whether an absolute protein level might influence the correlation with mRNA, the mean value of each protein (relative abundance) and the Spearman protein/mRNA correlation coefficients among all 85 samples were examined. No relationship between the protein abundance and the correlation coefficients was observed ($r = 0.039$; $p > 0.05$). A detailed analysis of separate subsets of proteins with differing levels of abundance (less than -0.0014, larger than -0.0014, or larger than 0.0077) also showed a lack of correlation between mRNA and protein expression among the 83 (50%), 82 (50%), and 41 (25%) of 165 total protein spots, respectively ($r = 0.016$, 0.08, and 0.172, respectively).

Stage-related Changes in the Protein/mRNA Correlation Coefficients—To determine whether the 21 genes (28 protein spots) showing a significant correlation between the protein and mRNA expression among all samples demonstrate changes in this relationship during tumor progression, the correlations were examined separately for stage I ($n = 57$) and

stage III ($n = 19$) lung adenocarcinomas (Table III). The number of non-neoplastic lung samples ($n = 9$) was insufficient for a separate correlation analysis of this group. Many of the protein spots represent one of several known protein isoforms for a given gene. The majority of genes (18/21) did not differ in the protein/mRNA correlation between stage I and stage III tumors indicating a similar regulatory relationship between the mRNA and protein spot. GRP-58, PSMC, SOD1, TPI1, and VIM, however, were found to demonstrate significant differences in the correlation coefficients between stage I and stage III lung adenocarcinomas. For GRP-58, PSMC, and VIM the change in the correlation coefficient was because of a relative increase in protein expression in stage III tumors. For SOD and TPI the change resulted from a relative decrease in expression of this specific protein in stage III tumors.

DISCUSSION

Relatively little is known about the regulatory mechanisms controlling the complex patterns of protein abundance and post-translational modification in tumors. Most reports concerning the regulation of protein translation have focused on

TABLE III
Stage-dependent analysis of protein-mRNA correlation coefficients

r, correlation coefficient. Values in boldface indicate a significant difference between stage I and stage III.

Spot	Gene name	r (Stage I)	r (Stage III)	Function
1874	AKR1B1	0.269	0.106	Carbohydrate metabolism; electron transporter
2524	ANXA1	0.184	0.572	Phospholipase inhibitor; signal transduction
0994	ANXA4	0.660	0.362	Phospholipase inhibitor
0963	ANXA5	0.241	0.390	Phospholipase inhibitor; calcium binding; phospholipid binding
1314	DJ-1	0.363	0.354	Signal transduction
1405	FTL	0.126	0.358	Iron storage protein
0855	GAPD	0.243	0.581	Carbohydrate metabolism (glycolysis regulation)
0350	GRP58	0.327	−0.087	Signal transduction; protein disulfide isomerase
0264	HNRPK	0.360	0.243	RNA-binding protein (RNA processing/modification)
1192	HSPB3	0.457	0.633	Heat shock protein
0523	KRT18	0.115	0.371	Structural protein
0439	KRT8	0.323	0.498	Structural protein
1492	LAP18	0.483	0.663	Signal transduction; cell growth and maintenance
1638	LGALS1	0.200	0.528	Apoptosis; cell adhesion; cell size control
1252	PSMC	0.253	0.060	Protein degradation
1104	SFN	0.465	0.475	Signal transduction (protein kinase C inhibitor)
1454	SOD1	0.352	0.079	Oxidoreductase
1203	TPI1	0.378	0.009	Carbohydrate metabolism
0857	TPM1	0.475	0.225	Structural protein (muscle); control of heart
0593	VIM	−0.054	0.556	Structural protein
0935	YWHAH	0.283	0.210	Signal transduction

one or several protein products (18). Cells et al. (19) found a good correlation between transcript and protein levels among 40 well resolved, abundant proteins using a proteomic and microarray study of bladder cancer. By comparing the mRNA and protein expression levels within the same tumor samples, we found that 17% (28/165) of the protein spots (21/98 genes) show a statistically significant correlation between mRNA and protein. These proteins appear to represent a diverse group of gene products and include those involved in signal transduction, carbohydrate metabolism, protein modification, cell structure, heat shock, and apoptosis. These results suggest that expression of this subset of 165 proteins is likely to be regulated at the transcriptional level in these tissues. The majority of the protein isoforms, however, did not correlate with mRNA levels, and thus their expression is regulated by other mechanisms. We also observed a subset of proteins that demonstrated a negative correlation with the mRNA expression values; for example α-haptoglobin demonstrated a strong negative correlation with its mRNA expression values. This may reflect negative feedback on the mRNA or the protein or the presence of other regulatory influences that are not understood currently.

Post-translational modification or processing will result in individual protein products of the same gene migrating to different locations on 2D-PAGE gels (20). Because the identity of all possible isoforms for each protein examined has not been characterized completely, this may influence the correlation analyses performed in this study. This is partly because of limitations of the 2D-PAGE and mass spectrometry technologies (21, 22). Potential inconsistencies between mRNA and protein correlations that have been reported may also be because of differences, even in the same gene, in the mech-

anisms of protein translation among different cells or as measured in different laboratories (23).

In this study, we examined 165 protein spots identified in lung adenocarcinomas. Ninety-six protein spots, representing the products of 29 genes, contained at least two protein isoforms. Nineteen of 96 protein spots, representing 12 genes, were shown to have a statistically significant correlation between their protein and mRNA expression, suggesting that the levels of these proteins reflects the transcription of the corresponding genes. Differences in protein/mRNA correlations were found among the individual isoforms of a given protein. For example, of the four OP18 isoforms, three showed a statistically significant correlation between the protein and mRNA expression levels. The lack of relationship for the one isoform, however, indicates that individual protein isoforms of the same gene product can be regulated differentially. This is not unexpected and likely reflects other post-translational mechanisms that can influence isoform abundance in tissues and cancer.

In addition to the analyses of the correlation of mRNA/protein within the same tumor samples, we also tested the global relationship between mRNA and the corresponding protein abundance across all 165 protein spots in the lung samples. A protein and mRNA average value for each gene was generated using all 85 lung tissues samples. We observed a very wide range of normalized average protein and mRNA values. The correlation coefficient generated using this average value data set was −0.025, and even for the 28 protein spots that showed a statistically significant correlation between individual mRNA and proteins, the correlation value was only −0.035. This suggests that it is not possible to predict overall protein expression levels based on average

A: Correlation of protein and mRNA across all 165 protein spots

r=-0.025

B: Correlation of protein and mRNA across 28 proteins with r> 0.244

r=-0.035

Fig. 3. The overall correlation of mRNA and protein levels across all 165 protein spots (A) and across 28 protein spots that contained individual *r* values larger than 0.244 (B) are shown. Each protein or mRNA mean value was calculated based on all 76 lung adenocarcinomas and nine nonneoplastic lung samples using quantitative 2D-PAGE and Affymetrix oligonucleotide microarrays. The Spearman correlation coefficients for the two data sets (A and B) were −0.025 and −0.035, respectively, indicating a lack of correlation if mean values for mRNA and protein for all samples is used.
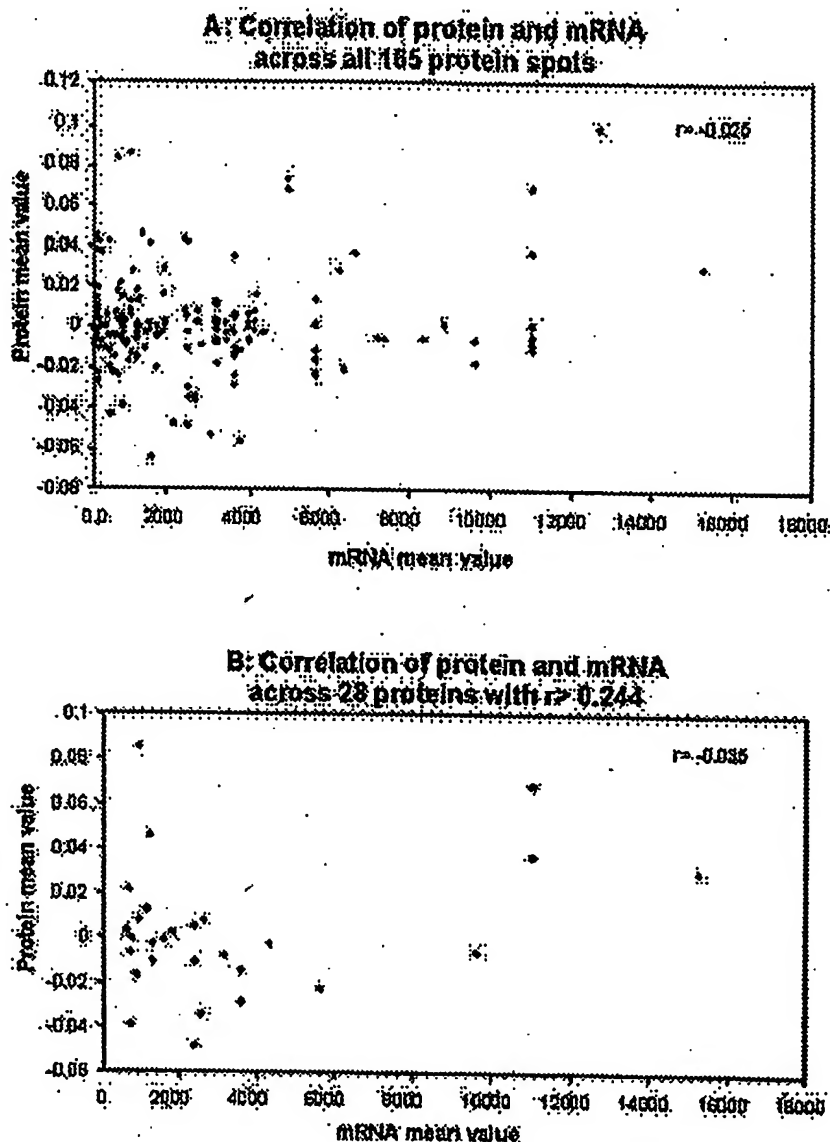
mRNA abundance in lung cancer samples. This conclusion is also supported by previous results from Anderson and Seilhamer (24), who examined 19 genes in human liver cells, and by Gygl et al. (25), who examined 106 genes in yeast. Both studies found a lack of correlation between mRNA and protein expression when average or overall levels were used.

A good correlation was reported when the 11 most abundant proteins were examined in yeast (25), suggesting that the level of protein abundance may be a factor that may influence the correlation between mRNA and protein. In the present study, a fairly wide range of mean protein values among 165 protein spots in lung adenocarcinomas was observed, and the correlation coefficients also varied from −0.467 to 0.442.

A comparison between the mean value of each protein and the correlation coefficient generated using all 85 tissue samples did not reveal a strong relationship between the overall protein abundance and the correlation coefficients ($r = 0.039$; $p > 0.05$). Detailed analysis of different subsets of protein abundance also failed to show a correlation between mRNA and protein expression. Thus in contrast to yeast, a relationship between mRNA/protein correlation coefficient and protein abundance in human lung adenocarcinomas was not observed.

The results of this study indicate that the level of protein abundance in lung adenocarcinomas is associated with the corresponding levels of mRNA in 17% (28 proteins) of the total 165 protein spots examined. This was substantially

higher than the amount predicted to result by chance alone (which was 5.1) and suggests that a transcriptional mechanism likely underlies the abundance of these proteins in lung adenocarcinomas. We also demonstrate that the expression of individual isoforms of the same protein may or may not correlate with the mRNA, indicating that separate and likely post-translational mechanisms account for the regulation of isoform abundance. These mechanisms may also account for the differences in the correlation coefficients observed between stage I and stage III tumors, indicating that specific protein isoforms show regulatory changes during tumor progression. Further studies in lung adenocarcinomas will examine the relationship between the expression of individual protein isoforms and specific clinical-pathological features of these tumors, such as the presence of angiolymphatic invasion, and nodal or pleural surface involvement. The potential to identify specific protein isoforms associated with biological behavior in lung adenocarcinomas would be of considerable interest and will add to our understanding of the regulation of gene products by transcriptional, translational, and post-translational mechanisms.

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MOLECULAR BIOLOGY OF
THE CELL
THIRD EDITION

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Front cover: The photograph shows a rat nerve cell in culture. It is labeled (*yellow*) with a fluorescent antibody that stains its cell body and dendritic processes. Nerve terminals (*green*) from other neurons (not visible), which have made synapses on the cell, are labeled with a different antibody. (Courtesy of Olaf Mundigl and Pietro de Camilli.)

Dedication page: Gavin Borden, late president of Garland Publishing, weathered in during his mid-1980s climb near Mount McKinley with MBoC author Bruce Alberts and famous mountaineer guide Mugs Stump (1940–1992).

Back cover: The authors, in alphabetical order, crossing Abbey Road in London on their way to lunch. Much of this third edition was written in a house just around the corner. (Photograph by Richard Olivier.)

extracts. If these minor cell proteins differ among cells to the same extent as the more abundant proteins, as is commonly assumed, only a small number of protein differences (perhaps several hundred) suffice to create very large differences in cell morphology and behavior.

A Cell Can Change the Expression of Its Genes in Response to External Signals [3]

Most of the specialized cells in a multicellular organism are capable of altering their patterns of gene expression in response to extracellular cues. If a liver cell is exposed to a glucocorticoid hormone, for example, the production of several specific proteins is dramatically increased. Glucocorticoids are released during periods of starvation or intense exercise and signal the liver to increase the production of glucose from amino acids and other small molecules; the set of proteins whose production is induced includes enzymes such as tyrosine aminotransferase, which helps to convert tyrosine to glucose. When the hormone is no longer present, the production of these proteins drops to its normal level.

Other cell types respond to glucocorticoids in different ways. In fat cells, for example, the production of tyrosine aminotransferase is reduced, while some other cell types do not respond to glucocorticoids at all. These examples illustrate a general feature of cell specialization—different cell types often respond in different ways to the same extracellular signal. Underlying this specialization are features that do not change, which give each cell type its permanently distinctive character. These features reflect the persistent expression of different sets of genes.

Gene Expression Can Be Regulated at Many of the Steps in the Pathway from DNA to RNA to Protein [4]

If differences between the various cell types of an organism depend on the particular genes that the cells express, at what level is the control of gene expression exercised? There are many steps in the pathway leading from DNA to protein, and all of them can in principle be regulated. Thus a cell can control the proteins it makes by (1) controlling when and how often a given gene is transcribed (**transcriptional control**), (2) controlling how the primary RNA transcript is spliced or otherwise processed (**RNA processing control**), (3) selecting which completed mRNAs in the cell nucleus are exported to the cytoplasm (**RNA transport control**), (4) selecting which mRNAs in the cytoplasm are translated by ribosomes (**translational control**), (5) selectively destabilizing certain mRNA molecules in the cytoplasm (**mRNA degradation control**), or (6) selectively activating, inactivating, or compartmentalizing specific protein molecules after they have been made (**protein activity control**) (Figure 9–2).

For most genes transcriptional controls are paramount. This makes sense because, of all the possible control points illustrated in Figure 9–2, only transcriptional control ensures that no superfluous intermediates are synthesized. In the
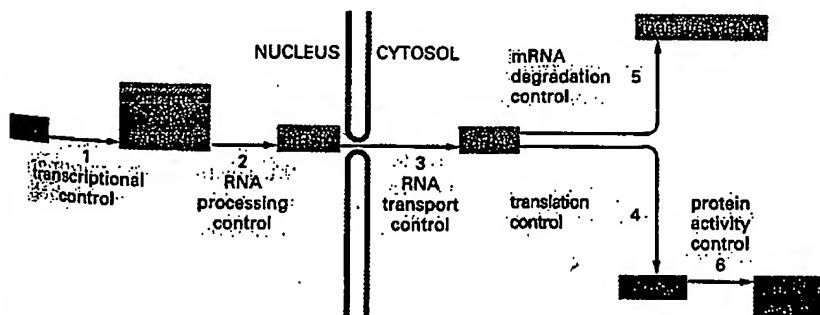
Figure 9–2 Six steps at which eucaryote gene expression can be controlled. Only controls that operate at steps 1 through 5 are discussed in this chapter. The regulation of protein activity (step 6) is discussed in Chapter 5; this includes reversible activation or inactivation by protein phosphorylation as well as irreversible inactivation by proteolytic degradation.

following sections we discuss the DNA and protein components that regulate the initiation of gene transcription. We return at the end of the chapter to the other ways of regulating gene expression.

Summary

The genome of a cell contains in its DNA sequence the information to make many thousands of different protein and RNA molecules. A cell typically expresses only a fraction of its genes, and the different types of cells in multicellular organisms arise because different sets of genes are expressed. Moreover, cells can change the pattern of genes they express in response to changes in their environment, such as signals from other cells. Although all of the steps involved in expressing a gene can in principle be regulated, for most genes the initiation of RNA transcription is the most important point of control.

# DNA-binding Motifs in Gene Regulatory Proteins [5]

How does a cell determine which of its thousands of genes to transcribe? As discussed in Chapter 8, the transcription of each gene is controlled by a regulatory region of DNA near the site where transcription begins. Some regulatory regions are simple and act as switches that are thrown by a single signal. Other regulatory regions are complex and act as tiny microprocessors, responding to a variety of signals that they interpret and integrate to switch the neighboring gene on or off. Whether complex or simple, these switching devices consist of two fundamental types of components: (1) short stretches of DNA of defined sequence and (2) *gene regulatory proteins* that recognize and bind to them.

We begin our discussion of gene regulatory proteins by describing how these proteins were discovered.

Gene Regulatory Proteins Were Discovered Using Bacterial Genetics [6]

Genetic analyses in bacteria carried out in the 1950s provided the first evidence of the existence of **gene regulatory proteins** that turn specific sets of genes on or off. One of these regulators, the *lambda repressor,* is encoded by a bacterial virus, *bacteriophage lambda.* The repressor shuts off the viral genes that code for the protein components of new virus particles and thereby enables the viral genome to remain a silent passenger in the bacterial chromosome, multiplying with the bacterium when conditions are favorable for bacterial growth (see Figure 6–80). The lambda repressor was among the first gene regulatory proteins to be characterized, and it remains one of the best understood, as we discuss later. Other bacterial regulators respond to nutritional conditions by shutting off genes encoding specific sets of metabolic enzymes when they are not needed. The *lac repressor,* for example, the first of these bacterial proteins to be recognized, turns off the production of the proteins responsible for lactose metabolism when this sugar is absent from the medium.

The first step toward understanding gene regulation was the isolation of mutant strains of bacteria and bacteriophage lambda that were unable to shut off specific sets of genes. It was proposed at the time, and later proved, that most of these mutants were deficient in proteins acting as specific repressors for these sets of genes. Because these proteins, like most gene regulatory proteins, are present in small quantities, it was difficult and time-consuming to isolate them. They were eventually purified by fractionating cell extracts on a series of standard chromatography columns (see pp. 166–169). Once isolated, the proteins were shown to bind to specific DNA sequences close to the genes that they

minor groove
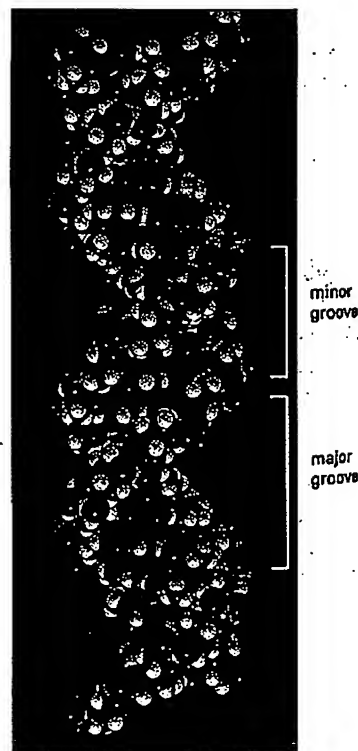
major groove

Figure 9–3 Double-helical structure of DNA. The major and minor grooves on the outside of the double helix are indicated. The atoms are colored as follows: carbon, *dark blue;* nitrogen, *light blue;* hydrogen, *white;* oxygen, *red;* phosphorus, *yellow.*

VERTEBRATE ANCESTOR DNA

methylation of most
CG sequences in
germ line

5' → RNA

many millions of years
of evolution

5' →

VERTEBRATE DNA

5' →

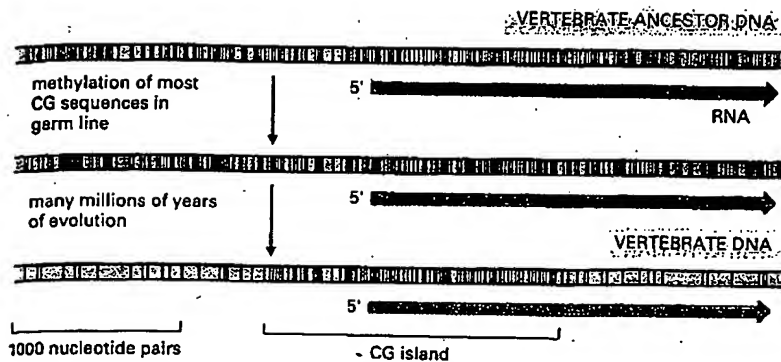1000 nucleotide pairs — CG island

Figure 9–71 A mechanism to explain both the marked deficiency of CG sequences and the presence of CG islands in vertebrate genomes. A *black line* marks the location of an unmethylated CG dinucleotide in the DNA sequence, while a *red line* marks the location of a methylated CG dinucleotide.

Summary

The many types of cells in animals and plants are created largely through mechanisms that cause different genes to be transcribed in different cells. Since many specialized animal cells can maintain their unique character when grown in culture, the gene regulatory mechanisms involved in creating them must be stable once established and heritable when the cell divides, endowing the cell with a memory of its developmental history. Procaryotes and yeasts provide unusually accessible model systems in which to study gene regulatory mechanisms, some of which may be relevant to the creation of specialized cell types in higher eucaryotes. One such mechanism involves a competitive interaction between two (or more) gene regulatory proteins, each of which inhibits the synthesis of the other; this can create a flip-flop switch that switches a cell between two alternative patterns of gene expression. Direct or indirect positive feedback loops, which enable gene regulatory proteins to perpetuate their own synthesis, provide a general mechanism for cell memory.

In eucaryotes gene transcription is generally controlled by combinations of gene regulatory proteins. It is thought that each type of cell in a higher eucaryotic organism contains a specific combination of gene regulatory proteins that ensures the expression of only those genes appropriate to that type of cell. A given gene regulatory protein may be expressed in a variety of circumstances and typically is involved in the regulation of many genes.

In addition to diffusible gene regulatory proteins, inherited states of chromatin condensation are also utilized by eucaryotic cells to regulate gene expression. In vertebrates DNA methylation also plays a part, mainly as a device to reinforce decisions about gene expression that are made initially by other mechanisms.

Posttranscriptional Controls

Although controls on the initiation of gene transcription are the predominant form of regulation for most genes, other controls can act later in the pathway from RNA to protein to modulate the amount of gene product that is made. Although these **posttranscriptional controls**, which operate after RNA polymerase has bound to the gene's promoter and begun RNA synthesis, are less common than *transcriptional control,* for many genes they are crucial. It seems that every step in gene expression that could be controlled in principle is likely to be regulated under some circumstances for some genes.

We consider the varieties of posttranscriptional regulation in temporal order, according to the sequence of events that might be experienced by an RNA molecule after its transcription has begun (Figure 9–72).
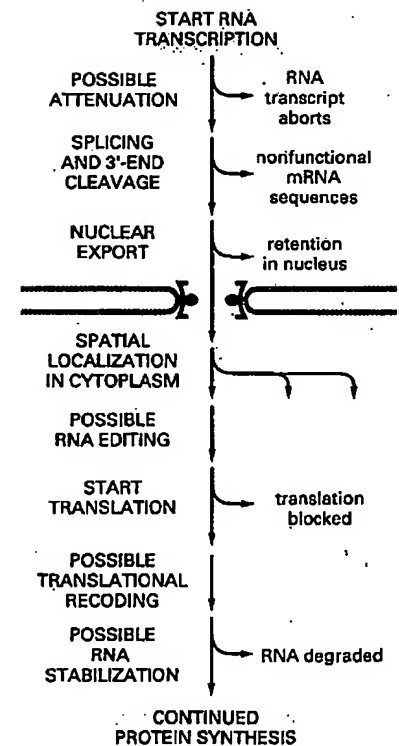

START RNA
TRANSCRIPTION

POSSIBLE ATTENUATION	RNA transcript aborts
SPLICING AND 3'-END CLEAVAGE	nonfunctional mRNA sequences
NUCLEAR EXPORT	retention in nucleus
SPATIAL LOCALIZATION IN CYTOPLASM	
POSSIBLE RNA EDITING	
START TRANSLATION	translation blocked
POSSIBLE TRANSLATIONAL RECODING	
POSSIBLE RNA STABILIZATION	RNA degraded

CONTINUED
PROTEIN SYNTHESIS

Figure 9–72 Possible posttranscriptional controls on gene expression. Only a few of these controls are likely to be used for any one gene.

CHAPTER 29

Regulation of transcription

Genes II (1997) CH 29, pp. 847-848.
Benjamin Lewin

The phenotypic differences that distinguish the various kinds of cells in a higher eukaryote are largely due to differences in the expression of genes that code for proteins, that is, those transcribed by RNA polymerase II. In principle, the expression of these genes might be regulated at any one of several stages. The concept of the "level of control" implies that gene expression is not necessarily an automatic process once it has begun. It could be regulated in a gene-specific way at any one of several sequential steps. We can distinguish (at least) five potential control points, forming the series:

Activation of gene structure
↓
Initiation of transcription
↓
Processing the transcript
↓
Transport to cytoplasm
↓
Translation of mRNA

The existence of the first step is implied by the discovery that genes may exist in either of two structural conditions. Relative to the state of most of the genome, genes are found in an "active" state in the cells in which they are expressed (see Chapter 27). The change of structure is distinct from the act of transcription, and indicates that the gene is "transcribable." This suggests that acquisition of the "active" structure must be the first step in gene expression.

Transcription of a gene in the active state is

controlled at the stage of initiation, that is, by the interaction of RNA polymerase with its promoter. This is now becoming susceptible to analysis in the *in vitro* systems (see Chapter 28). For most genes, this is a major control point: probably it is the most common level of regulation.

There is at present no evidence for control at subsequent stages of transcription in eukaryotic cells, for example, via antitermination mechanisms.

The primary transcript is modified by capping at the 5′ end, and usually also by polyadenylation at the 3′ end. Introns must be spliced out from the transcripts of interrupted genes. The mature RNA must be exported from the nucleus to the cytoplasm. Regulation of gene expression by selection of sequences at the level of nuclear RNA might involve any or all of these stages, but the one for which we have most evidence concerns changes in splicing: some genes are expressed by means of alternative splicing patterns whose regulation controls the type of protein product (see Chapter 30).

Finally, the translation of an mRNA in the cytoplasm can be specifically controlled. There is little evidence for the employment of this mechanism in adult somatic cells, but it does occur in some embryonic situations, as described in Chapter 7. The mechanism is presumed to involve the blocking of initiation of translation of some mRNAs by specific protein factors.

But having acknowledged that control of gene expression can occur at multiple stages, and that production of RNA cannot inevitably be equated with production of protein; it is clear

that the overwhelming majority of regulatory events occur at the initiation of transcription. Regulation of tissue-specific gene transcription lies at the heart of eukaryotic differentiation; indeed, we see examples in Chapter 38 in which proteins that regulate embryonic development prove to be transcription factors. A regulatory transcription factor serves to provide common control of a large number of target genes, and we seek to answer two questions about this mode of regulation: what identifies the common target genes to the transcription factor; and how is the activity of the transcription factor itself regulated in response to intrinsic or extrinsic signals?

Response elements identify genes under common regulation

The principle that emerges from characterizing groups of genes under common control is that *they share a promoter element that is recognized by a regulatory transcription factor.* An element that causes a gene to respond to such a factor is called a response element; examples are the HSE (heat shock response element), GRE (glucocorticoid response element), SRE (serum response element).

The properties of some inducible transcription factors and the elements that they recognize are summarized in Table 29.1. Response elements have the same general characteristics as upstream elements of promoters or enhancers. They contain short consensus sequences, and copies of the response elements found in different genes are closely related, but not necessarily identical. The region bound by the factor extends for a short distance on either side of the consensus sequence. In promoters, the elements are not present at fixed distances from the startpoint, but are usually <200 bp upstream of it. The presence of a single element usually is sufficient to confer the regulatory response, but sometimes there are multiple copies.

Response elements may be located in promoters or in enhancers. Some types of elements are typically found in one rather than the other: usually an HSE is found in a promoter, while a GRE is found in an enhancer. We assume that all response elements function by the same general principle. *A gene is regulated by a sequence at the promoter or enhancer that is recognized by a specific protein. The protein functions as a transcription factor needed for RNA polymerase to initiate. Active protein is available only under conditions when the gene is to be expressed; its absence means that the promoter is not activated by this particular circuit.*

An example of a situation in which many genes are controlled by a single factor is provided by the heat shock response. This is common to a wide range of prokaryotes and eukaryotes and involves multiple controls of gene expression: an increase in temperature turns off transcription of some genes, turns and transcription of the heat shock genes, and causes changes in the translation of mRNAs. The control of the heat shock genes illustrates the differences between prokaryotic and eukaryotic modes of control. In bacteria, a new sigma factor is synthesized that directs RNA polymerase holoenzyme to recognize an alter-

Table 29.1 Inducible transcription factors bind to response elements that identify groups of promoters or enhancers subject to coordinate control.

Regulatory Agent	Module	Consensus	Factor
Heat shock	HSE	CNNGAANNTCCNNG	HSTF
Glucocorticoid	GRE	TGGTACAAATGTTCT	Receptor
Phorbol ester	TRE	TGACTCA	AP1
Serum	SRE	CCATATTAGG	SRF

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Research

Prostate stem cell antigen (PSCA) expression in human prostate cancer tissues and its potential role in prostate carcinogenesis and progression of prostate cancer

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Abstract

Background: Prostate stem cell antigen (PSCA) is a recently defined homologue of the Thy-1/Ly-6 family of glycosylphosphatidylinositol (GPI)-anchored cell surface antigens. The purpose of the present study was to examine the expression status of PSCA protein and mRNA in clinical specimens of human prostate cancer (Pca) and to validate it as a potential molecular target for diagnosis and treatment of Pca.

Materials and Methods: Immunohistochemical (IHC) and *in situ* hybridization (ISH) analyses of PSCA expression were simultaneously performed on paraffin-embedded sections from 20 benign prostatic hyperplasia (BPH), 20 prostatic intraepithelial neoplasm (PIN) and 48 prostate cancer (Pca) tissues, including 9 androgen-independent prostate cancers. The level of PSCA expression was semiquantitatively scored by assessing both the percentage and intensity of PSCA-positive staining cells in the specimens. Then compared PSCA expression between BPH, PIN and Pca tissues and analysed the correlations of PSCA expression level with pathological grade, clinical stage and progression to androgen-independence in Pca.

Results: In BPH and low grade PIN, PSCA protein and mRNA staining were weak or negative and less intense and uniform than that seen in HGPIN and Pca. There were moderate to strong PSCA protein and mRNA expression in 8 of 11 (72.7%) HGPIN and in 40 of 48 (83.4%) Pca specimens examined by IHC and ISH analyses, with statistical significance compared with BPH (20%) and low grade PIN (22.2%) samples (p < 0.05, respectively). The expression level of PSCA increased with high Gleason grade, advanced stage and progression to androgen-independence (p < 0.05, respectively). In addition, IHC and ISH staining showed a high degree of correlation between PSCA protein and mRNA overexpression.

Conclusions: Our data demonstrate that PSCA as a new cell surface marker is overexpressed by a majority of human Pca. PSCA expression correlates positively with adverse tumor characteristics, such as increasing pathological grade (poor cell differentiation), worsening clinical stage and androgen-independence, and speculatively with prostate carcinogenesis. PSCA protein overexpression results from upregulated transcription of PSCA mRNA. PSCA may have prognostic utility and may be a promising molecular target for diagnosis and treatment of Pca.

Introduction

Prostate cancer (Pca) is the second leading cause of cancer-related death in American men and is becoming a common cancer increasing in China. Despite recently great progress in the diagnosis and management of localized disease, there continues to be a need for new diagnostic markers that can accurately discriminate between indolent and aggressive variants of Pca. There also continues to be a need for the identification and characterization of potential new therapeutic targets on Pca cells. Current diagnostic and therapeutic modalities for recurrent and metastatic Pca have been limited by a lack of specific target antigens of Pca.

Although a number of prostate-specific genes have been identified (i.e. prostate specific antigen, prostatic acid phosphatase, glandular kallikrein 2), the majority of these are secreted proteins not ideally suited for many immunological strategies. So, the identification of new cell surface antigens is critical to the development of new diagnostic and therapeutic approaches to the management of Pca.

Reiter RE et al [1] reported the identification of prostate stem cell antigen (PSCA), a cell surface antigen that is predominantly prostate specific. The PSCA gene encodes a 123 amino acid glycoprotein, with 30% homology to stem cell antigen 2 (Sca 2). Like Sca-2, PSCA also belongs to a member of the Thy-1/Ly-6 family and is anchored by a glycosylphosphatidylinositol (GPI) linkage. mRNA *in situ* hybridization (ISH) localized PSCA expression in normal prostate to the basal cell epithelium, the putative stem cell compartment of prostatic epithelium, suggesting that PSCA may be a marker of prostate stem/progenitor cells.

In order to examine the status of PSCA protein and mRNA expression in human Pca and validate it as a potential diagnostic and therapeutic target for Pca, we used immunohistochemistry (IHC) and *in situ* hybridization (ISH) simultaneously, and conducted PSCA protein and mRNA expression analyses in paraffin-embedded tissue specimens of benign prostatic hyperplasia (BPH, n = 20), prostate intraepithelial neoplasm (PIN, n = 20) and prostate cancer (Pca, n = 48). Furthermore, we evaluated the possible correlation of PSCA expression level with Pca tumorigenesis, grade, stage and progression to androgen-independence.

Materials and methods

Tissue samples

All of the clinical tissue specimens studied herein were obtained from 80 patients of 57–84 years old by prostatectomy, transurethral resection of prostate (TURP) or biopsies. The patients were classified as 20 cases of BPH, 20 cases of PIN, 40 cases of primary Pca, including 9 patients with recurrent Pca and a history of androgen ablation therapy (orchiectomy and/or hormonal therapy), who were referred to as androgen-independent prostate cancers. Eight specimens were harvested from these androgen-independent Pca patients prior to androgen ablation treatment. Each tissue sample was cut into two parts, one was fixed in 10% formalin for IHC and the other treated with 4% paraformaldehyde/0.1 M PBS PH 7.4 in 0.1% DEPC for 1 h for ISH analysis, and then embedded in paraffin. All paraffin blocks examined were then cut into 5 μm sections and mounted on the glass slides specific for IHC and ISH respectively in the usual fashion. H&E-stained section of each Pca was evaluated and assigned a Gleason score by the experienced urological pathologist at our institution based on the criteria of Gleason score [2]. The Gleason sums are summarized in Table 1. Clinical staging was performed according to Jewett-whitmore-prout staging system, as shown in Table 2. In the category of PIN, we graded the specimens into two groups, i.e. low grade PIN (grade I – II) and high grade PIN (HGPIN, grade III) on the basis of literatures [3,4].

Immunohistochemical (IHC) analysis

Briefly, tissue sections were deparaffinized, dehydrated, and subjected to microwaving in 10 mmol/L citrate buffer, PH 6.0 (Boshide, Wuhan, China) in a 900 W oven for 5 min to induce epitope retrieval. Slides were allowed to cool at room temperature for 30 min. A primary mouse antibody specific to human PSCA (Boshide, Wuhan, China) with a 1:100 dilution was applied to incubate with the slides at room temperature for 2 h. Labeling was detected by sequentially adding biotinylated secondary antibodies and strepavidin-peroxidase, and localized using 3,3'-diaminobenzidine reaction. Sections were then counterstained with hematoxylin. Substitution of the primary antibody with phosphate-buffered-saline (PBS) served as a negative-staining control.

mRNA in situ hybridization (ISH)

Five-μm-thick tissue sections were deparaffinized and dehydrated, then digested in pepsin solution (4 mg/ml in 3% citric acid) for 20 min at 37.5°C, and further processed for ISH. Digoxigenin-labeled sense and antisense human PSCA RNA probes (obtained from Boshide, Wuhan, China) were hybridized to the sections at 48°C overnight. The posthybridization wash with a high stringency was performed sequentially at 37°C in 2 × standard saline citrate (SSC) for 10 min, in 0.5 × SSC for 15 min and in 0.2 × SSC for 30 min. The slides were then incubated to biotinylated mouse anti-digoxigenin antibody at 37.5°C for 1 h followed by washing in 1 × PBS for 20 min at room temperature, and then to strepavidin-peroxidase at 37.5°C for 20 min followed by washing in 1 × PBS for 15 min at room temperature. Subsequently, the slides were developed with diaminobenzidine and then coun-

Table 1: Correlation of PSCA expression with Gleason score

Gleason score	Intensity × frequency	
	0–6 (%)	9 (%)
2–4	5 (83)	1 (17)
5–7	19 (79)	5 (21)
8–10	5 (28)	13 (72)

Table 2: Correlation of PSCA expression with clinical stage

Tumor stage	Intensity × frequency	
	0–6 (%)	9 (%)
≤B	27 (67.5)	13 (32.5)
≥C	2 (25)	6 (75)

terstained with hematoxylin to localize the hybridization signals. Sections hybridized with the sense control probes routinely did not show any specific hybridization signal above background. All slides were hybridized with PBS to substitute for the probes as a negative control.

Scoring methods

To determine the correlation between the results of PSCA immunostaining and mRNA *in situ* hybridization, the same scoring manners are taken in the present study for PSCA protein staining by IHC and PSCA mRNA staining by ISH. Each slide was read and scored by two independently experienced urological pathologists using Olympus BX-41 light microscopes. The evaluation was done in a blinded fashion. For each section, five areas of similar grade were analyzed semiquantitatively for the fraction of cells staining. Fifty percent of specimens were randomly chosen and rescored to determine the degree of interobserver and intraobserver concordance. There was greater than 95% intra- and interobserver agreement.

The intensity of PSCA expression evaluated microscopically was graded on a scale of 0 to 3+ with 3 being the highest expression observed (0, no staining; 1+, mildly intense; 2+, moderately intense; 3+, severely intense). The staining density was quantified as the percentage of cells staining positive for PSCA with the primary antibody or hybridization probe, as follows: 0 = no staining; 1 = positive staining in <25% of the sample; 2 = positive staining in 25%–50% of the sample; 3 = positive staining in >50%

of the sample. Intensity score (0 to 3+) was multiplied by the density score (0–3) to give an overall score of 0–9 [1,5]. In this way, we were able to differentiate specimens that may have had focal areas of increased staining from those that had diffuse areas of increased staining [6]. The overall score for each specimen was then categorically assigned to one of the following groups: 0 score, negative expression; 1–2 scores, weak expression; 3–6 scores, moderate expression; 9 score, strong expression.

Statistical analysis

Intensity and density of PSCA protein and mRNA expression in BPH, PIN and Pca tissues were compared using the Chi-square and Student's *t*-test. Univariate associations between PSCA expression and Gleason score, clinical stage and progression to androgen-independence were calculated using Fisher's Exact Test. For all analyses, p < 0.05 was considered statistically significant.

Results

PSCA expression in BPH

In general, PSCA protein and mRNA were expressed weakly in individual samples of BPH. Some areas of prostate expressed weak levels (composite score 1–2), whereas other areas were completely negative (composite score 0). Four cases (20%) of BPH had moderate expression of PSCA protein and mRNA (composite score 4–6) by IHC and ISH. In 2/20 (10%) BPH specimens, PSCA mRNA expression was moderate (composite score 3–6), but PSCA protein expression was weak (composite score

2) in one and negative (composite score 0) in the other. PSCA expression was localized to the basal and secretory epithelial cells, and prostatic stroma was almost negative staining for PSCA protein and mRNA in all cases examined.

PSCA expression in PIN

In this study, we detected weak or negative expression of PSCA protein and mRNA (≤ 2 scores) in 7 of 9 (77.8%) low grade PIN and in 2 of 11 (18.2%) HGPIN, and moderate expression (3–6 scores) in the rest 2 low grade PIN and 5 of 11 (45.5%) HGPIN. One HGPIN with moderate PSCA mRNA expression (6 score) was found weak staining for PSCA protein (2 score) by IHC. Strong PSCA protein and mRNA expression (9 score) were detected in the remaining 3 of 11 (27.3%) HGPIN. There was a statistically significant difference of PSCA protein and mRNA expression levels observed between HGPIN and BPH ($p < 0.05$), but no statistical difference reached between low grade PIN and BPH ($p > 0.05$).

PSCA expression in Pca

In order to determine if PSCA protein and mRNA can be detected in prostate cancers and if PSCA expression levels are increased in malignant compared with benign glands, Forty-eight paraffin-embedded Pca specimens were analysed by IHC and ISH. It was shown that 19 of 48 (39.6%) Pca samples stained very strongly for PSCA protein and mRNA with a score of 9 and another 21 (43.8%) specimens displayed moderate staining with scores of 4–6 (Figure 1). In addition, 4 specimens with moderate to strong PSCA mRNA expression (scores of 4–9) had weak protein staining (a score of 2) by IHC analyses. Overall, Pca expressed a significantly higher level of PSCA protein and mRNA than any other specimen category in this study ($p < 0.05$, compared with BPH and PIN respectively). The result demonstrates that PSCA protein and mRNA are overexpressed by a majority of human Pca.

Correlation of PSCA expression with Gleason score in Pca
Using the semi-quantitative scoring method as described in Materials and Methods, we compared the expression level of PSCA protein and mRNA with Gleason grade of Pca, as shown in Table 1. Prostate adenocarcinomas were graded by Gleason score as 2–4 scores = well-differentiation, 5–7 scores = moderate-differentiation and 8–10 scores = poor-differentiation [7]. Seventy-two percent of Gleason scores 8–10 prostate cancers had very strong staining of PSCA compared to 21% with Gleason scores 5–7 and 17% with 2–4 respectively, demonstrating that poorly differentiated Pca had significantly stronger expression of PSCA protein and mRNA than moderately and well differentiated tumors ($p < 0.05$). As depicted in Figure 1, IHC and ISH analyses showed that PSCA protein and mRNA expression in several cases of poorly differen-

tiated Pca were particularly prominent, with more intense and uniform staining. The results indicate that PSCA expression increases significantly with higher tumor grade in human Pca.

Correlation of PSCA expression with clinical stage in Pca
With regards to PSCA expression in every stage of Pca, we showed the results in Table 2. Seventy-five percent of locally advanced and node positive cancers (i.e. C-D stages) expressed statistically high levels of PSCA versus 32.5% that were organ confined (i.e. A-B stages) ($p < 0.05$). The data demonstrate that PSCA expression increases significantly with advanced tumor stage in human Pca.

Correlation of PSCA expression with androgen-independent progression of Pca
All 9 specimens of androgen-independent prostate cancers stained positive for PSCA protein and mRNA. Eight specimens were obtained from patients managed prior to androgen ablation therapy. Seven of eight (87.5%) of these androgen-independent prostate cancers were in the strongest staining category (score = 9), compared with three out of eight (37.5%) of patients with androgen-dependent cancers ($p < 0.05$). The results demonstrate that PSCA expression increases significantly with progression to androgen-independence of human Pca.

It is evident from the results above that within a majority of human prostate cancers the level of PSCA protein and mRNA expression correlates significantly with increasing grade, worsening stage and progression to androgen-independence.

Correlation of PSCA immunostaining and mRNA in situ hybridization
In all 88 specimens surveyed herein, we compared the results of PSCA IHC staining with mRNA ISH analysis. Positive staining areas and its intensity and density scores evaluated by IHC were identical to those seen by ISH in 79 of 88 (89.8%) specimens (18/20 BPH, 19/20 PIN and 42/48 Pca respectively). Importantly, 27/27 samples with PSCA mRNA composite scores of 0–2, 32/36 samples with scores of 3–6 and 22/24 samples with a score of 9 also had PSCA protein expression scores of 0–2, 3–6 and 9 respectively. However, in 5 samples with PSCA mRNA overall scores of 3–6 and in 2 with scores of 9 there were less or negative PSCA protein expression (i.e. scores of 0–4), suggesting that this may reflect posttranscriptional modification of PSCA or that the epitopes recognized by PSCA mAb may be obscured in some cancers. The data demonstrate that the results of PSCA immunostaining were consistent with those of mRNA ISH analysis, showing a high degree of correlation between PSCA protein and mRNA expression.
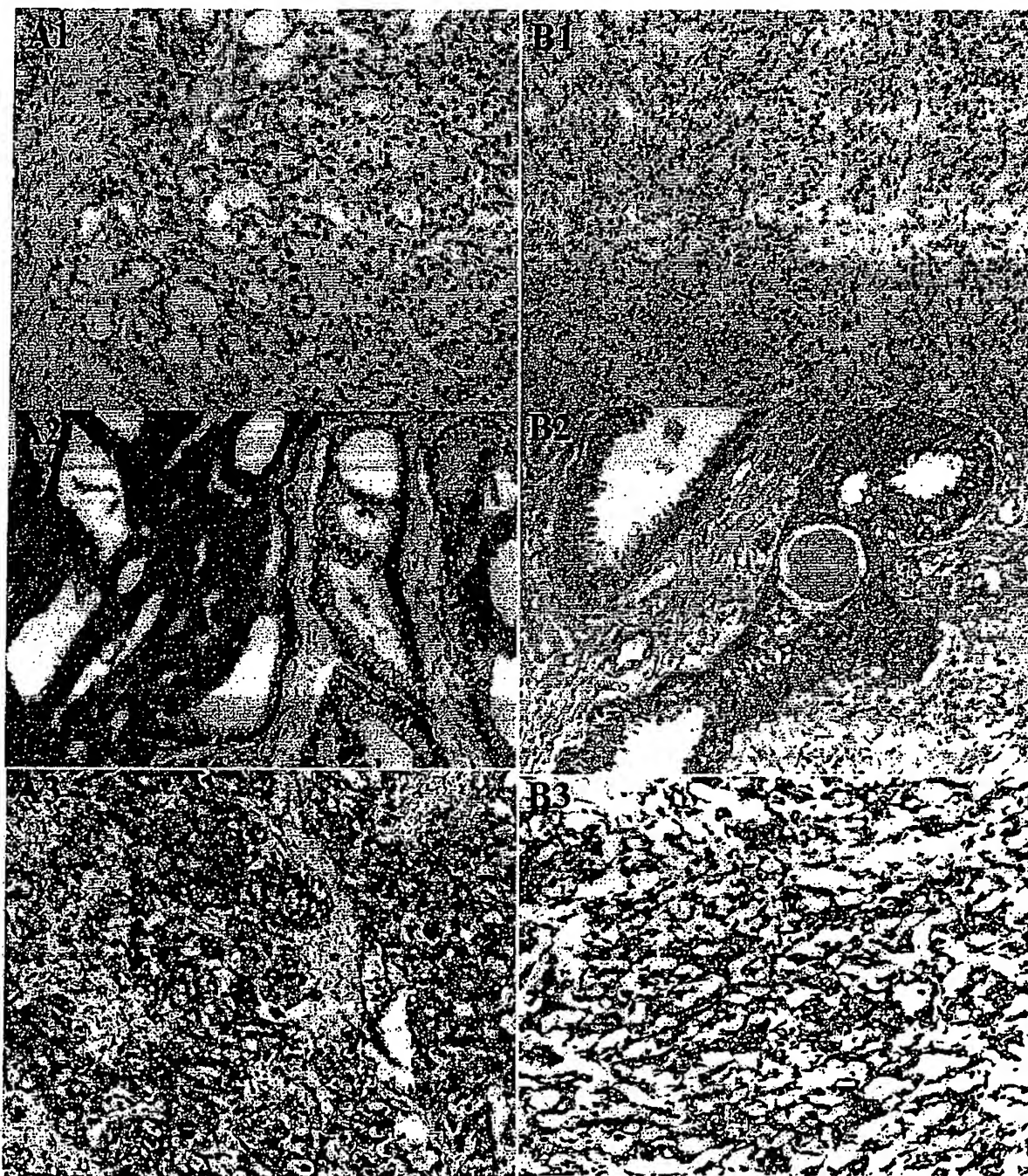
Figure 1
Representatives of PSCA IHC and ISH staining in Pca (A. IHC staining, B. ISH staining, ×200 magnification). A_1, B_1: negative control of IHC and ISH. PBS replacing the primary antibody (A_1) and hybridization with a sense PSCA probe (B_1) showed no background staining. A_2, B_2: a moderately differentiated Pca (Gleason score = 3+3 = 6) with moderate staining (composite score = 6) in all malignant cells; A_2: IHC shows not only cell surface but also apparent cytoplasmic staining of PSCA protein. A_3, B_3: a poorly differentiated Pca (Gleason score = 4+4 = 8) with very strong staining (composite score = 9) in all malignant cells.

Discussion

PSCA is homologous to a group of cell surface proteins that mark the earliest phase of hematopoietic development. PSCA mRNA expression is prostate-specific in normal male tissues and is highly up-regulated in both androgen-dependent and-independent Pca xenografts (LAPC-4 tumors). We hypothesize that PSCA may play a role in Pca tumorigenesis and progression, and may serve as a target for Pca diagnosis and treatment. In this study, IHC and ISH showed that in general there were weak or absent PSCA protein and mRNA expression in BPH and low grade PIN tissues. However, PSCA protein and mRNA are widely expressed in HGPIN, the putative precursor of invasive Pca, suggesting that up-regulation of PSCA is an early event in prostate carcinogenesis. Recently, Reiter RE et al [1], using ISH analysis, reported that 97 of 118 (82%) HGPIN specimens stained strongly positive for PSCA mRNA. A very similar finding was seen on mouse PSCA (mPSCA) expression in mouse HGPIN tissues by Tran C. P et al [8]. These data suggest that PSCA may be a new marker associated with transformation of prostate cells and tumorigenesis.

We observed that PSCA protein and mRNA are highly expressed in a large percentage of human prostate cancers, including advanced, poorly differentiated, androgen-independent and metastatic cases. Fluorescence-activated cell sorting and confocal/ immunofluorescent studies demonstrated cell surface expression of PSCA protein in Pca cells [9]. Our IHC expression analysis of PSCA shows not only cell surface but also apparent cytoplasmic staining of PSCA protein in Pca specimens (Figure 1). One possible explanation for this is that anti-PSCA antibody can recognize PSCA peptide precursors that reside in the cytoplasm. Also, it is possible that the positive staining that appears in the cytoplasm is actually from the overlying cell membrane [5]. These data seem to indicate that PSCA is a novel cell surface marker for human Pca.

Our results show that elevated level of PSCA expression correlates with high grade (i.e. poor differentiation), increased tumor stage and progression to androgen-independence of Pca. These findings support the original IHC analyses by Gu Z et al [9], who reported that PSCA protein expressed in 94% of primary Pca and the intensity of PSCA protein expression increased with tumor grade, stage and progression to androgen-independence. Our results also collaborate the recent work of Han KR et al [10], in which the significant association between high PSCA expression and adverse prognostic features such as high Gleason score, seminal vesicle invasion and capsular involvement in Pca was found. It is suggested that PSCA overexpression may be an adverse predictor for recurrence, clinical progression or survival of Pca. Hara H et al [11] used RT-PCR detection of PSA, PSMA and PSCA in 1

ml of peripheral blood to evaluate Pca patients with poor prognosis. The results showed that among 58 PCa patients, each PCR indicated the prognostic value in the hierarchy of PSCA>PSA>PSMA RT-PCR, and extraprostatic cases with positive PSCA PCR indicated lower disease-progression-free survival than those with negative PSCA PCR, demonstrating that PSCA can be used as a prognostic factor. Dubey P et al [12] reported that elevated numbers of PSCA + cells correlate positively with the onset and development of prostate carcinoma over a long time span in the prostates of the TRAMP and PTEN +/- models compared with its normal prostates. Taken together with our present findings, in which PSCA is overexpressed from HGPIN to almost frank carcinoma, it is reasonable and possible to use increased PSCA expression level or increased numbers of PSCA-positive cells in the prostate samples as a prognostic marker to predict the potential onset of this cancer. These data raise the possibility that PSCA may have diagnostic utility or clinical prognostic value in human Pca.

The cause of PSCA overexpression in Pca is not known. One possible mechanism is that it may result from PSCA gene amplification. In humans, PSCA is located on chromosome 8q24.2 [1], which is often amplified in metastatic and recurrent Pca and considered to indicate a poor prognosis [13-15]. Interestingly, PSCA is in close proximity to the c-myc oncogene, which is amplified in >20% of recurrent and metastatic prostate cancers [16,17]. Reiter RE et al [18] reported that PSCA and MYC gene copy numbers were co-amplified in 25% of tumors (five out of twenty), demonstrating that PSCA overexpression is associated with PSCA and MYC coamplification in Pca. Gu Z et al [9] recently reporeted that in 102 specimens available to compare the results of PSCA immunostaining with their previous mRNA ISH analysis, 92 (90.2%) had identically positive areas of PSCA protein and mRNA expression. Taken together with our findings, in which we detected moderate to strong expression of PSCA protein and mRNA in 34 of 40 (85%) Pca specimens examined simultaneously by IHC and ISH analyses, it is demonstrated that PSCA protein and mRNA overexpressed in human Pca, and that the increased protein level of PSCA was resulted from the upregulated transcription of its mRNA.

At present, the regulation mechanisms of human PSCA expression and its biological function are yet to be elucidated. PSCA expression may be regulated by multiple factors [18]. Watabe T et al [19] reported that transcriptional control is a major component regulating PSCA expression levels. In addition, induction of PSCA expression may be regulated or mediated through cell-cell contact and protein kinase C (PKC) [20]. Homologues of PSCA have diverse activities, and have themselves been involved in

carcinogenesis. Signalling through SCA-2 has been demonstrated to prevent apoptosis in immature thymocytes [21]. Thy-1 is involved in T cell activation and transducts signals through src-like tyrosine kinases [22]. Ly-6 genes have been implicated both in tumorigenesis and in cell-cell adhesion [23-25]. Cell-cell or cell-matrix interaction is critical for local tumor growth and spread to distal sites. From its restricted expression in basal cells of normal prostate and its homology to SCA-2, PSCA may play a role in stem/progenitor cell function, such as self-renewal (i.e. anti-apoptosis) and/or proliferation [1]. Taken together with the results in the present study, we speculate that PSCA may play a role in tumorigenesis and clinical progression of Pca through affecting cell transformation and proliferation. From our results, it is also suggested that PSCA as a new cell surface antigen may have a number of potential uses in the diagnosis, therapy and clinical prognosis of human Pca. PSCA overexpression in prostate biopsies could be used to identify patients at high risk to develop recurrent or metastatic disease, and to discriminate cancers from normal glands in prostatectomy samples. Similarly, the detection of PSCA-overexpressing cells in bone marrow or peripheral blood may identify and predict metastatic progression better than current assays, which identify only PSA-positive or PSMA-positive prostate cells.

In summary, we have shown in this study that PSCA protein and mRNA are maintained in expression from HGPIN through all stages of Pca in a majority of cases, which may be associated with prostate carcinogenesis and correlate positively with high tumor grade (poor cell differentiation), advanced stage and androgen-independent progression. PSCA protein overexpression is due to the upregulation of its mRNA transcription. The results suggest that PSCA may be a promising molecular marker for the clinical prognosis of human Pca and a valuable target for diagnosis and therapy of this tumor.

Competing interests
None declared.

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Review

Translation Initiation in Cancer: A Novel Target for Therapy[1]

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Abstract

Translation initiation is regulated in response to nutrient availability and mitogenic stimulation and is coupled with cell cycle progression and cell growth. Several alterations in translational control occur in cancer. Variant mRNA sequences can alter the translational efficiency of individual mRNA molecules, which in turn play a role in cancer biology. Changes in the expression or availability of components of the translational machinery and in the activation of translation through signal transduction pathways can lead to more global changes, such as an increase in the overall rate of protein synthesis and translational activation of the mRNA molecules involved in cell growth and proliferation. We review the basic principles of translational control, the alterations encountered in cancer, and selected therapies targeting translation initiation to help elucidate new therapeutic avenues.

Introduction

The fundamental principle of molecular therapeutics in cancer is to exploit the differences in gene expression between cancer cells and normal cells. With the advent of cDNA array technology, most efforts have concentrated on identifying differences in gene expression at the level of mRNA, which can be attributable either to DNA amplification or to differences in transcription. Gene expression is quite complicated, however, and is also regulated at the level of mRNA stability, mRNA translation, and protein stability.

The power of translational regulation has been best recognized among developmental biologists, because transcription does not occur in early embryogenesis in eukaryotes. For example, in *Xenopus*, the period of transcriptional quiescence continues until the embryo reaches midblastula transition, the 4000-cell stage. Therefore, all necessary mRNA molecules are transcribed during oogenesis and stockpiled in a translationally inactive, masked form. The mRNA are translationally activated at appropriate times during oocyte maturation, fertilization, and early embryogenesis and thus, are under strict translational control.

Translation has an established role in cell growth. Basically, an increase in protein synthesis occurs as a consequence of mitogenesis. Until recently, however, little was known about the alterations in mRNA translation in cancer, and much is yet to be discovered about their role in the development and progression of cancer. Here we review the basic principles of translational control, the alterations encountered in cancer, and selected therapies targeting translation initiation to elucidate potential new therapeutic avenues.

Basic Principles of Translational Control
Mechanism of Translation Initiation

Translation initiation is the main step in translational regulation. Translation initiation is a complex process in which the initiator tRNA and the 40S and 60S ribosomal subunits are recruited to the 5' end of a mRNA molecule and assembled by eukaryotic translation initiation factors into an 80S ribosome at the start codon of the mRNA (Fig. 1). The 5' end of eukaryotic mRNA is capped, i.e., contains the cap structure m^7GpppN (7-methyl-guanosine-triphospho-5'-ribonucleoside). Most translation in eukaryotes occurs in a cap-dependent fashion, i.e., the cap is specifically recognized by the eIF4E,[3] which binds the 5' cap. The eIF4F translation initiation complex is then formed by the assembly of eIF4E, the RNA helicase eIF4A, and eIF4G, a scaffolding protein that mediates the binding of the 40S ribosomal subunit to the mRNA molecule through interaction with the eIF3 protein present on the 40S ribosome. eIF4A and eIF4B participate in melting the secondary structure of the 5' UTR of the mRNA. The 43S initiation complex (40S/eIF2/Met-tRNA/GTP complex) scans the mRNA in a 5'→3' direction until it encounters an AUG start codon. This start codon is then base-paired to the anticodon of initiator tRNA, forming the 48S initiation complex. The initiation factors are then displaced from the 48S complex, and the 60S ribosome joins to form the 80S ribosome.

Unlike most eukaryotic translation, translation initiation of certain mRNAs, such as the picornavirus RNA, is cap independent and occurs by internal ribosome entry. This mechanism does not require eIF4E. Either the 43S complex can bind the initiation codon directly through interaction with the IRES in the 5' UTR such as in the encephalomyocarditis virus, or it can

[3] The abbreviations used are: eIF4E, eukaryotic initiation factor 4E; UTR, untranslated region; IRES, internal ribosome entry site; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; S6K, ribosomal p70 S6 kinase; mTOR, mammalian target of rapamycin; ATM, ataxia telangiectasia mutated; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog deleted from chromosome 10; PP2A, protein phosphatase 2A; TGF-β3, transforming growth factor-β3; PAP, poly(A) polymerase; EPA, eicosopentaenoic acid; mda-7, melanoma differentiation-associated gene 7.
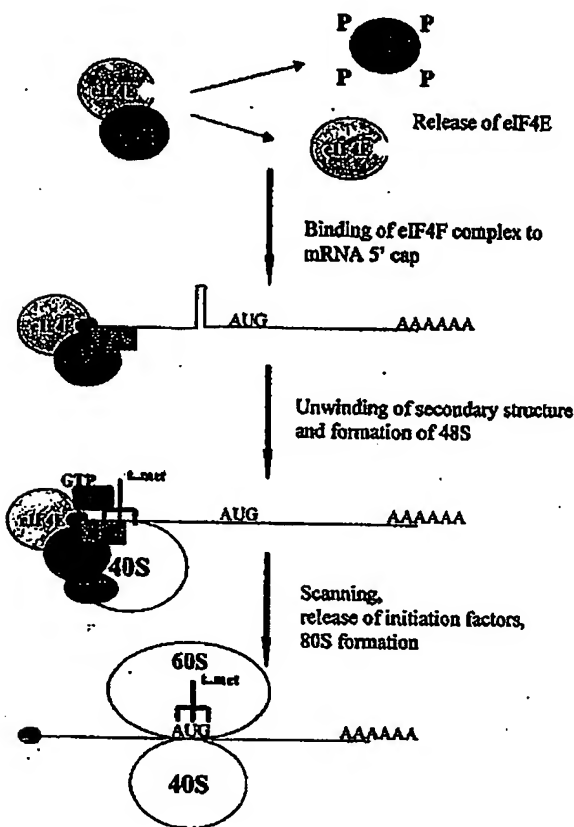
Fig. 1. Translation initiation in eukaryotes. The 4E-BPs are hyperphosphorylated to release eIF4E so that it can interact with the 5' cap, and the eIF4F initiation complex is assembled. The interaction of poly(A) binding protein with the initiation complex and circularization of the mRNA is not depicted in the diagram. The secondary structure of the 5' UTR is melted, the 40S ribosomal subunit is bound to eIF3, and the ternary complex consisting of eIF2, GTP, and the Met-tRNA are recruited to the mRNA. The ribosome scans the mRNA in a 5'→3' direction until an AUG start codon is found in the appropriate sequence context. The initiation factors are released, and the large ribosomal subunit is recruited.

initially attach to the IRES and then reach the initiation codon by scanning or transfer, as is the case with the poliovirus (1).

Regulation of Translation Initiation

Translation initiation can be regulated by alterations in the expression or phosphorylation status of the various factors involved. Key components in translational regulation that may provide potential therapeutic targets follow.

eIF4E. eIF4E plays a central role in translation regulation. It is the least abundant of the initiation factors and is considered the rate-limiting component for initiation of cap-dependent translation. eIF4E may also be involved in mRNA splicing, mRNA 3' processing, and mRNA nucleocytoplasmic transport (2). eIF4E expression can be increased at the transcriptional level in response to serum or growth factors (3). eIF4E overexpression may cause preferential translation of mRNAs containing excessive secondary structure in their 5' UTR that are normally discriminated against by the trans-

lational machinery and thus are inefficiently translated (4–7). As examples of this, overexpression of eIF4E promotes increased translation of vascular endothelial growth factor, fibroblast growth factor-2, and cyclin D1 (2, 8, 9).

Another mechanism of control is the regulation of eIF4E phosphorylation. eIF4E phosphorylation is mediated by the mitogen-activated protein kinase-interacting kinase 1, which is activated by the mitogen-activated pathway activating extracellular signal-related kinases and the stress-activated pathway acting through p38 mitogen-activated protein kinase (10–13). Several mitogens, such as serum, platelet-derived growth factor, epidermal growth factor, insulin, angiotensin II, src kinase overexpression, and ras overexpression, lead to eIF4E phosphorylation (14). The phosphorylation status of eIF4E is usually correlated with the translational rate and growth status of the cell; however, eIF4E phosphorylation has also been observed in response to some cellular stresses when translational rates actually decrease (15). Thus, further study is needed to understand the effects of eIF4E phosphorylation on eIF4E activity.

Another mechanism of regulation is the alteration of eIF4E availability by the binding of eIF4E to the eIF4E-binding proteins (4E-BP, also known as PHAS-I). 4E-BPs compete with eIF4G for a binding site in eIF4E. The binding of eIF4E to the best characterized eIF4E-binding protein, 4E-BP1, is regulated by 4E-BP1 phosphorylation. Hypophosphorylated 4E-BP1 binds to eIF4E, whereas 4E-BP1 hyperphosphorylation decreases this binding. Insulin, angiotensin, epidermal growth factor, platelet-derived growth factor, hepatocyte growth factor, nerve growth factor, insulin-like growth factors I and II, interleukin 3, granulocyte-macrophage colony-stimulating factor + steel factor, gastrin, and the adenovirus have all been reported to induce phosphorylation of 4E-BP1 and to decrease the ability of 4E-BP1 to bind eIF4E (15, 16). Conversely, deprivation of nutrients or growth factors results in 4E-BP1 dephosphorylation, an increase in eIF4E binding, and a decrease in cap-dependent translation.

p70 S6 Kinase. Phosphorylation of ribosomal 40S protein S6 by S6K is thought to play an important role in translational regulation. S6K −/− mouse embryonic cells proliferate more slowly than do parental cells, demonstrating that S6K has a positive influence on cell proliferation (17). S6K regulates the translation of a group of mRNAs possessing a 5' terminal oligopyrimidine tract (5' TOP) found at the 5' UTR of ribosomal protein mRNAs and other mRNAs coding for components of the translational machinery. Phosphorylation of S6K is regulated in part based on the availability of nutrients (18, 19) and is stimulated by several growth factors, such as platelet-derived growth factor and insulin-like growth factor I (20).

eIF2α Phosphorylation. The binding of the initiator tRNA to the small ribosomal unit is mediated by translation initiation factor eIF2. Phosphorylation of the α-subunit of eIF2 prevents formation of the eIF2/GTP/Met-tRNA complex and inhibits global protein synthesis (21, 22). eIF2α is phosphorylated under a variety of conditions, such as viral infection, nutrient deprivation, heme deprivation, and apoptosis (22). eIF2α is phosphorylated by heme-regulated inhibitor, nutrient-regulated protein kinase, and the IFN-induced, double-stranded RNA-activated protein kinase (PKR; Ref. 23).

The mTOR Signaling Pathway. The macrolide antibiotic rapamycin (Siralimus; Wyeth-Ayerst Research, Collegeville, PA) has been the subject of intensive study because it inhibits signal transduction pathways involved in T-cell activation. The rapamycin-sensitive component of these pathways is mTOR (also called FRAP or RAFT1). mTOR is the mammalian homologue of the yeast TOR proteins that regulate G_1 progression and translation in response to nutrient availability (24). mTOR is a serine-threonine kinase that modulates translation initiation by altering the phosphorylation status of 4E-BP1 and S6K (Fig. 2; Ref. 25).

4E-BP1 is phosphorylated on multiple residues. mTOR phosphorylates the Thr-37 and Thr-46 residues of 4E-BP1 in vitro (26); however, phosphorylation at these sites is not associated with a loss of eIF4E binding. Phosphorylation of Thr-37 and Thr-46 is required for subsequent phosphorylation at several COOH-terminal, serum-sensitive sites; a combination of these phosphorylation events appears to be needed to inhibit the binding of 4E-BP1 to eIF4E (25). The product of the ATM gene, p38/MSK1 pathway, and protein kinase Cσ also play a role in 4E-BP1 phosphorylation (27–29).

S6K and 4E-BP1 are also regulated, in part, by PI3K and its downstream protein kinase Akt. PTEN is a phosphatase that negatively regulates PI3K signaling. PTEN null cells have constitutively active of Akt, with increased S6K activity and S6 phosphorylation (30). S6K activity is inhibited both by PI3K inhibitors wortmannin and LY294002 and by mTOR inhibitor rapamycin (24). Akt phosphorylates Ser-2448 in mTOR in vitro, and this site is phosphorylated upon Akt activation in vivo (31–33). Thus, mTOR is regulated by the PI3K/Akt pathway; however, this does not appear to be the only mode of regulation of mTOR activity. Whether the PI3K pathway also regulates S6K and 4E-BP1 phosphorylation independent of mTOR is controversial.

Interestingly, mTOR autophosphorylation is blocked by wortmannin but not by rapamycin (34). This seeming inconsistency suggests that mTOR-responsive regulation of 4E-BP1 and S6K activity occurs through a mechanism other than intrinsic mTOR kinase activity. An alternate pathway for 4E-BP1 and S6K phosphorylation by mTOR activity is by the inhibition of a phosphatase. Treatment with calyculin A, an inhibitor of phosphatases 1 and 2A, reduces rapamycin-induced dephosphorylation of 4E-BP1 and S6K by rapamycin (35). PP2A interacts with full-length S6K but not with a S6K mutant that is resistant to dephosphorylation resulting from rapamycin. mTOR phosphorylates PP2A in vitro; however, how this process alters PP2A activity is not known. These results are consistent with the model that phosphorylation of a phosphatase by mTOR prevents dephosphorylation of 4E-BP1 and S6K, and conversely, that nutrient deprivation and rapamycin block inhibition of the phosphatase by mTOR.

Polyadenylation. The poly(A) tail in eukaryotic mRNA is important in enhancing translation initiation and mRNA stability. Polyadenylation plays a key role in regulating gene expression during oogenesis and early embryogenesis. Some mRNA that are translationally inactive in the oocyte are polyadenylated concomitantly with translational activation in oocyte maturation, whereas other mRNAs that are translationally active during oogenesis are deadenylated and trans-
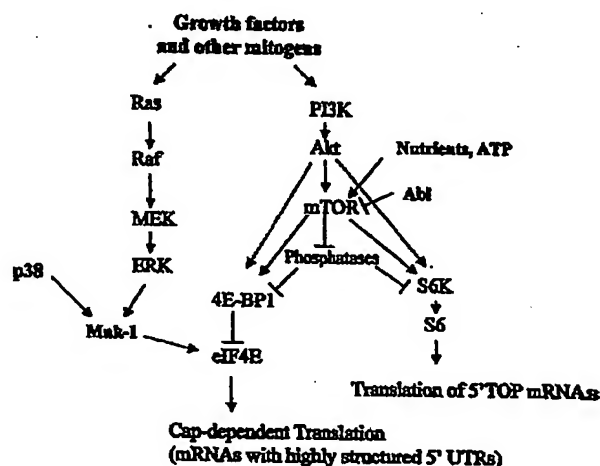

Fig. 2. Regulation of translation initiation by signal transduction pathways. Signaling via p38, extracellular signal-related kinase, PI3K, and mTOR can all activate translation initiation.

lationally silenced (36–38). Thus, control of poly(A) tail synthesis is an important regulatory step in gene expression. The 5' cap and poly(A) tail are thought to function synergistically to regulate mRNA translational efficiency (39, 40).

RNA Packaging. Most RNA-binding proteins are assembled on a transcript at the time of transcription, thus determining the translational fate of the transcript (41). A highly conserved family of Y-box proteins is found in cytoplasmic messenger ribonucleoprotein particles, where the proteins are thought to play a role in restricting the recruitment of mRNA to the translational machinery (41–43). The major mRNA-associated protein, YB-1, destabilizes the interaction of eIF4E and the 5' mRNA cap in vitro, and overexpression of YB-1 results in translational repression in vivo (44). Thus, alterations in RNA packaging can also play an important role in translational regulation.

Translation Alterations Encountered in Cancer

Three main alterations at the translational level occur in cancer: variations in mRNA sequences that increase or decrease translational efficiency, changes in the expression or availability of components of the translational machinery, and activation of translation through aberrantly activated signal transduction pathways. The first alteration affects the translation of an individual mRNA that may play a role in carcinogenesis. The second and third alterations can lead to more global changes, such as an increase in the overall rate of protein synthesis, and the translational activation of several mRNA species.

Variations in mRNA Sequence

Variations in mRNA sequence affect the translational efficiency of the transcript. A brief description of these variations and examples of each mechanism follow.

Mutations. Mutations in the mRNA sequence, especially in the 5' UTR, can alter its translational efficiency, as seen in the following examples.

c-myc. Saito et al. proposed that translation of full-length c-myc is repressed, whereas in several Burkitt lymphomas that have deletions of the mRNA 5' UTR, translation of c-myc is more efficient (45). More recently, it was reported that the 5' UTR of c-myc contains an IRES, and thus c-myc translation can be initiated by a cap-independent as well as a cap-dependent mechanism (46, 47). In patients with multiple myeloma, a C→T mutation in the c-myc IRES was identified (48) and found to cause an enhanced initiation of translation via internal ribosomal entry (49).

BRCA1. A somatic point mutation (117 G→C) in position −3 with respect to the start codon of the BRCA1 gene was identified in a highly aggressive sporadic breast cancer (50). Chimeric constructs consisting of the wild-type or mutated BRCA1 5' UTR and a downstream luciferase reporter demonstrated a decrease in the translational efficiency with the 5' UTR mutation.

Cyclin-dependent Kinase Inhibitor 2A. Some inherited melanoma kindreds have a G→T transversion at base −34 of cyclin-dependent kinase inhibitor-2A, which encodes a cyclin-dependent kinase 4/cyclin-dependent kinase 6 kinase inhibitor important in G_1 checkpoint regulation (51). This mutation gives rise to a novel AUG translation initiation codon, creating an upstream open reading frame that competes for scanning ribosomes and decreases translation from the wild-type AUG.

Alternate Splicing and Alternate Transcription Start Sites. Alterations in splicing and alternate transcription sites can lead to variations in 5' UTR sequence, length, and secondary structure, ultimately impacting translational efficiency.

ATM. The ATM gene has four noncoding exons in its 5' UTR that undergo extensive alternative splicing (52). The contents of 12 different 5' UTRs that show considerable diversity in length and sequence have been identified. These divergent 5' leader sequences play an important role in the translational regulation of the ATM gene.

mdm. In a subset of tumors, overexpression of the oncoprotein mdm2 results in enhanced translation of the mdm2 mRNA. Use of different promoters leads to two mdm2 transcripts that differ only in their 5' leaders (53). The longer 5' UTR contains two upstream open reading frames, and this mRNA is loaded with ribosomes inefficiently compared with the short 5' UTR.

BRCA1. In a normal mammary gland, BRCA1 mRNA is expressed with a shorter leader sequence (5'UTRa), whereas in sporadic breast cancer tissue, BRCA1 mRNA is expressed with a longer leader sequence (5' UTRb); the translational efficiency of transcripts containing 5' UTRb is 10 times lower than that of transcripts containing 5' UTRa (54).

TGF-β3. TGF-β3 mRNA includes a 1.1-kb 5' UTR, which exerts an inhibitory effect on translation. Many human breast cancer cell lines contain a novel TGF-β3 transcript with a 5' UTR that is 870 nucleotides shorter and has a 7-fold greater translational efficiency than the normal TGF-β3 mRNA (55).

Alternate Polyadenylation Sites. Multiple polyadenylation signals leading to the generation of several transcripts with differing 3' UTR have been described for several mRNA species, such as the RET proto-oncogene (56), ATM gene (52), tissue inhibitor of metalloproteinases-3 (57), RHOA

proto-oncogene (58), and calmodulin-1 (59). Although the effect of these alternate 3' UTRs on translation is not yet known, they may be important in RNA-protein interactions that affect translational recruitment. The role of these alterations in cancer development and progression is unknown.

Alterations in the Components of the Translation Machinery

Alterations in the components of translation machinery can take many forms.

Overexpression of eIF4E. Overexpression of eIF4E causes malignant transformation in rodent cells (60) and the deregulation of HeLa cell growth (61). Polunovsky et al. (62) found that eIF4E overexpression substitutes for serum and individual growth factors in preserving viability of fibroblasts, which suggests that eIF4E can mediate both proliferative and survival signaling.

Elevated levels of eIF4E mRNA have been found in a broad spectrum of transformed cell lines (63). eIF4E levels are elevated in all ductal carcinoma in situ specimens and invasive ductal carcinomas, compared with benign breast specimens evaluated with Western blot analysis (64, 65). Preliminary studies suggest that this overexpression is attributable to gene amplification (66).

There are accumulating data suggesting that eIF4E overexpression can be valuable as a prognostic marker. eIF4E overexpression was found in a retrospective study to be a marker of poor prognosis in stages I to III breast carcinoma (67). Verification of the prognostic value of eIF4E in breast cancer is now under way in a prospective trial (67). However, in a different study, eIF4E expression was correlated with the aggressive behavior of non-Hodgkin's lymphomas (68). In a prospective analysis of patients with head and neck cancer, elevated levels of eIF4E in histologically tumor-free surgical margins predicted a significantly increased risk of local-regional recurrence (9). These results all suggest that eIF4E overexpression can be used to select patients who might benefit from more aggressive systemic therapy. Furthermore, the head and neck cancer data suggest that eIF4E overexpression is a field defect and can be used to guide local therapy.

Alterations in Other Initiation Factors. Alterations in a number of other initiation factors have been associated with cancer. Overproduction of eIF4G, similar to eIF4E, leads to malignant transformation in vitro (69). eIF-2α is found in increased levels in bronchioloalveolar carcinomas of the lung (3). Initiation factor eIF-4A1 is overexpressed in melanoma (70) and hepatocellular carcinoma (71). The p40 subunit of translation initiation factor 3 is amplified and overexpressed in breast and prostate cancer (72), and the eIF3-p110 subunit is overexpressed in testicular seminoma (73). The role that overexpression of these initiation factors plays on the development and progression of cancer, if any, is not known.

Overexpression of S6K. S6K is amplified and highly overexpressed in the MCF7 breast cancer cell line, compared with normal mammary epithelium (74). In a study by Barlund et al. (74), S6K was amplified in 59 of 668 primary breast tumors, and a statistically significant association was observed between amplification and poor prognosis.

Overexpression of PAP. PAP catalyzes 3' poly(A) synthesis. PAP is overexpressed in human cancer cells compared with normal and virally transformed cells (75). PAP enzymatic activity in breast tumors has been correlated with PAP protein levels (76) and, in mammary tumor cytosols, was found to be an independent factor for predicting survival (76). Little is known, however, about how PAP expression or activity affects the translational profile.

Alterations in RNA-binding Proteins. Even less is known about alterations in RNA packaging in cancer. Increased expression and nuclear localization of the RNA-binding protein YB-1 are indicators of a poor prognosis for breast cancer (77), non-small cell lung cancer (78), and ovarian cancer (79). However, this effect may be mediated at least in part at the level of transcription, because YB-1 increases chemoresistance by enhancing the transcription of a multidrug resistance gene (80).

Activation of Signal Transduction Pathways

Activation of signal transduction pathways by loss of tumor suppressor genes or overexpression of certain tyrosine kinases can contribute to the growth and aggressiveness of tumors. An important mutant in human cancers is the tumor suppressor gene *PTEN*, which leads to the activation of the PI3K/Akt pathway. Activation of PI3K and Akt induces the oncogenic transformation of chicken embryo fibroblasts. The transformed cells show constitutive phosphorylation of S6K and of 4E-BP1 (81). A mutant Akt that retains kinase activity but does not phosphorylate S6K or 4E-BP1 does not transform fibroblasts, which suggests a correlation between the oncogenicity of PI3K and Akt and the phosphorylation of S6K and 4E-BP1 (81).

Several tyrosine kinases such as platelet-derived growth factor, insulin-like growth factor, HER2/neu, and epidermal growth factor receptor are overexpressed in cancer. Because these kinases activate downstream signal transduction pathways known to alter translation initiation, activation of translation is likely to contribute to the growth and aggressiveness of these tumors. Furthermore, the mRNA for many of these kinases themselves are under translational control. For example, HER2/neu mRNA is translationally controlled both by a short upstream open reading frame that represses HER2/neu translation in a cell type-independent manner and by a distinct cell type-dependent mechanism that increases translational efficiency (82). HER2/neu translation is different in transformed and normal cells. Thus, it is possible that alterations at the translational level can in part account for the discrepancy between *HER2/neu* gene amplification detected by fluorescence *in situ* hybridization and protein levels detected by immunohistochemical assays.

Translation Targets of Selected Cancer Therapy

Components of the translation machinery and signal pathways involved in the activation of translation initiation represent good targets for cancer therapy.

Targeting the mTOR Signaling Pathway: Rapamycin and Tumstatin

Rapamycin inhibits the proliferation of lymphocytes. It was initially developed as an immunosuppressive drug for organ

transplantation. Rapamycin with FKBP 12 (FK506-binding protein, M_r 12,000) binds to mTOR to inhibit its function.

Rapamycin causes a small but significant reduction in the initiation rate of protein synthesis (83). It blocks cell growth in part by blocking S6 phosphorylation and selectively suppressing the translation of 5' TOP mRNAs, such as ribosomal proteins, and elongation factors (83–85). Rapamycin also blocks 4E-BP1 phosphorylation and inhibits cap-dependent but not cap-independent translation (17, 86).

The rapamycin-sensitive signal transduction pathway, activated during malignant transformation and cancer progression, is now being studied as a target for cancer therapy (87). Prostate, breast, small cell lung, glioblastoma, melanoma, and T-cell leukemia are among the cancer lines most sensitive to the rapamycin analogue CCI-779 (Wyeth-Ayerst Research; Ref. 87). In rhabdomyosarcoma cell lines, rapamycin is either cytostatic or cytocidal, depending on the p53 status of the cell; p53 wild-type cells treated with rapamycin arrest in the G_1 phase and maintain their viability, whereas p53 mutant cells accumulate in G_1 and undergo apoptosis (88, 89). In a recently reported study using human primitive neuroectodermal tumor and medulloblastoma models, rapamycin exhibited more cytotoxicity in combination with cisplatin and camptothecin than as a single agent. In vivo, CCI-779 delayed growth of xenografts by 160% after 1 week of therapy and 240% after 2 weeks. A single high-dose administration caused a 37% decrease in tumor volume. Growth inhibition in vivo was 1.3 times greater, with cisplatin in combination with CCI-779 than with cisplatin alone (90). Thus, preclinical studies suggest that rapamycin analogues are useful as single agents and in combination with chemotherapy.

Rapamycin analogues CCI-779 and RAD001 (Novartis, Basel, Switzerland) are now in clinical trials. Because of the known effect of rapamycin on lymphocyte proliferation, a potential problem with rapamycin analogues is immunosuppression. However, although prolonged immunosuppression can result from rapamycin and CCI-779 administered on continuous-dose schedules, the immunosuppressive effects of rapamycin analogues resolve in ~24 h after therapy (91). The principal toxicities of CCI-779 have included dermatological toxicity, myelosuppression, infection, mucositis, diarrhea, reversible elevations in liver function tests, hyperglycemia, hypokalemia, hypocalcemia, and depression (87, 92–94). Phase II trials of CCI-779 have been conducted in advanced renal cell carcinoma and in stage III/IV breast carcinoma patients who failed with prior chemotherapy. In the results reported in abstract form, although there were no complete responses, partial responses were documented in both renal cell carcinoma and in breast carcinoma (94, 95). Thus, CCI-779 has documented preliminary clinical activity in a previously treated, unselected patient population.

Active investigation is under way into patient selection for mTOR inhibitors. Several studies have found an enhanced efficacy of CCI-779 in PTEN-null tumors (30, 96). Another study found that six of eight breast cancer cell lines were responsive to CCI-779, although only two of these lines lacked PTEN (97) There was, however, a positive correlation between Akt activation and CCI-779 sensitivity (97). This correlation suggests that activation of the PI3K-Akt pathway,

regardless of whether it is attributable to a PTEN mutation or to overexpression of receptor tyrosine kinases, makes cancer cell amenable to mTOR-directed therapy. In contrast, lower levels of the target of mTOR, 4E-BP1, are associated with rapamycin resistance; thus, a lower 4E-BP1/eIF4E ratio may predict rapamycin resistance (98).

Another mode of activity for rapamycin and its analogues appears to be through inhibition of angiogenesis. This activity may be both through direct inhibition of endothelial cell proliferation as a result of mTOR inhibition in these cells or by inhibition of translation of such proangiogenic factors as vascular endothelial growth factor in tumor cells (99, 100).

The angiogenesis inhibitor tumstatin, another anticancer drug currently under study, was also found recently to inhibit translation in endothelial cells (101). Through a requisite interaction with integrin, tumstatin inhibits activation of the PI3K/Akt pathway and mTOR in endothelial cells and prevents dissociation of eIF4E from 4E-BP1, thereby inhibiting cap-dependent translation. These findings suggest that endothelial cells are especially sensitive to therapies targeting the mTOR-signaling pathway.

Targeting eIF2α: EPA, Clotrimazole, mda-7, and Flavonoids

EPA is an n-3 polyunsaturated fatty acid found in the fish-based diets of populations having a low incidence of cancer (102). EPA inhibits the proliferation of cancer cells (103), as well as in animal models (104, 105). It blocks cell division by inhibiting translation initiation (105). EPA releases Ca^{2+} from intracellular stores while inhibiting their refilling, thereby activating PKR. PKR, in turn phosphorylates and inhibits eIF2α, resulting in the inhibition of protein synthesis at the level of translation initiation. Similarly, clotrimazole, a potent antiproliferative agent *in vitro* and *in vivo*, inhibits cell growth through depletion of Ca^{2+} stores, activation of PKR, and phosphorylation of eIF2α (106). Consequently, clotrimazole preferentially decreases the expression of cyclins A, E, and D1, resulting in blockage of the cell cycle in G_1.

mda-7 is a novel tumor suppressor gene being developed as a gene therapy agent. Adenoviral transfer of *mda-7* (Ad-mda7) induces apoptosis in many cancer cells including breast, colorectal, and lung cancer (107–109). Ad-mda7 also induces and activates PKR, which leads to phosphorylation of eIF2α and induction of apoptosis (110).

Flavonoids such as genistein and quercetin suppress tumor cell growth. All three mammalian eIF2α kinases, PKR, heme-regulated inhibitor, and PERK/PEK, are activated by flavonoids, with phosphorylation of eIF2α and inhibition of protein synthesis (111).

Targeting eIF4A and eIF4E: Antisense RNA and Peptides

Antisense expression of eIF4A decreases the proliferation rate of melanoma cells (112). Sequestration of eIF4E by overexpression of 4E-BP1 is proapoptotic and decreases tumorigenicity (113, 114). Reduction of eIF4E with antisense RNA decreases soft agar growth, increases tumor latency, and increases the rates of tumor doubling times (7). Antisense eIF4E RNA treat-

ment also reduces the expression of angiogenic factors (115) and has been proposed as a potential adjuvant therapy for head and neck cancers, particularly when elevated eIF4E is found in surgical margins. Small molecule inhibitors that bind the eIF4G/4E-BP1-binding domain of eIF4E are proapoptotic (116) and are also being actively pursued.

Exploiting Selective Translation for Gene Therapy

A different therapeutic approach that takes advantage of the enhanced cap-dependent translation in cancer cells is the use of gene therapy vectors encoding suicide genes with highly structured 5′ UTR. These mRNA would thus be at a competitive disadvantage in normal cells and not translate well, whereas in cancer cells, they would translate more efficiently. For example, the introduction of the 5′ UTR of fibroblast growth factor-2 5′ to the coding sequence of *herpes simplex virus type-1 thymidine kinase* gene, allows for selective translation of *herpes simplex virus type-1 thymidine kinase* gene in breast cancer cell lines compared with normal mammary cell lines and results in selective sensitivity to ganciclovir (117).

Toward the Future

Translation is a crucial process in every cell. However, several alterations in translational control occur in cancer. Cancer cells appear to need an aberrantly activated translational state for survival, thus allowing the targeting of translation initiation with surprisingly low toxicity. Components of the translational machinery, such as eIF4E, and signal transduction pathways involved in translation initiation, such mTOR, represent promising targets for cancer therapy. Inhibitors of the mTOR have already shown some preliminary activity in clinical trials. It is possible that with the development of better predictive markers and better patient selection, response rates to single-agent therapy can be improved. Similar to other cytostatic agents, however, mTOR inhibitors are most likely to achieve clinical utility in combination therapy. In the interim, our increasing understanding of translation initiation and signal transduction pathways promise to lead to the identification of new therapeutic targets in the near future.

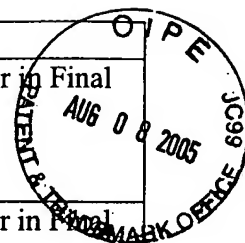### Acknowledgments

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Tab	Reference	Submitted	Entered
1	First Declaration of J. Christopher Grimaldi	Originally submitted as Exhibit 1 of 1st Amendment and Response	Entered by Examiner in Final Office Action
2	Second Declaration of J. Christopher Grimaldi	Originally submitted as Exhibit 2 of 1st Amendment and Response	Entered by Examiner in Final Office Action
3	Declaration of Paul Polakis, Ph.D.	Originally submitted as Exhibit 3 of 1st Amendment and Response	Entered by Examiner in Final Office Action
4	Bruce Alberts, *et al.*, Molecular Biology of the Cell (4th ed. 2002)	Originally submitted as Exhibit 4 of 1st Amendment and Response	Entered by Examiner in Final Office Action
5	Chen *et al.* (*Molecular and Cellular Proteomics*, 1:304-313 (2002)		Cited by Examiner in Advisory Action
6	Bruce Alberts, *et al.*, *Molecular Biology of the Cell* (3rd ed. 1994)	Originally submitted with Appellants' After Final Amendment as Exhibit 1	Entered by Examiner in Advisory Action
7	Benjamin Lewin, Genes VI (1997)	Originally submitted with Appellants' After Final Amendment as Exhibit 3	Entered by Examiner in Advisory Action
8	Zhigang *et al.*, World Journal of Surgical Oncology 2:13, 2004	Originally submitted with Appellants' After Final Amendment as Exhibit 4	Entered by Examiner in Advisory Action
9	Meric *et al.*, Molecular Cancer Therapeutics, vol. 1, 971-979 (2002)	Originally submitted with Appellants' After Final Amendment as Exhibit 5	Entered by Examiner in Advisory Action
10	U.S. Patent No. 6,414,117	Originally submitted with Appellants' After Final Amendment as Exhibit 6	Entered by Examiner in Advisory Action
11	U.S. Patent No. 6,124,433	Originally submitted with Appellants' After Final Amendment as Exhibit 7	Entered by Examiner in Advisory Action
12	U.S. Patent No. 6,156,500	Originally submitted with Appellants' After Final Amendment as Exhibit 8	Entered by Examiner in Advisory Action
13	U.S. Patent No. 6,562,343	Originally submitted with Appellants' After Final Amendment as Exhibit 9	Entered by Examiner in Advisory Action

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